Elucidating the Role of the Hypoxia-Protective Gene CPT1C in Cell Biology and Carcinogenesis

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Ines Lohse

aus

Deutschland

Promotionskomitee

Prof. Dr. Michael Hengartner (Vorsitz)

Dr. Dr. Kathrin Zaugg (Leitung der Dissertation)

Prof. Dr. Wilhelm Krek

Prof. Dr. Tak Mak

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Abstract

The Carnitine Palmitoyltransferase 1c (CPT1C) has recently been identified as a p53-regulated brain-specific Carnitine Palmitoyltransferase (CPT) 1-family member. The CPT1-family of proteins regulates fatty acid oxidation at the step of fatty acid import into the mitochondria. Three CPT1 genes, demonstrating generalized tissue specificity, have been identified in mammals. The physiological roles of CPT1A, the prominent CPT1 gene in liver, and CPT1B, the prominent CPT1 gene in muscle, are well established due to their role in pathogenicity. In contrast, the physiological role of CPT1C remains elusive.

Although CPT1C expression is found in neurons throughout the entire brain, CPT1C expression is enriched in brain regions involved in the regulation of peripheral energy expenditure such as hypothalamus, amygdala and hippocampus. Hypothalamic feeding centers contain nutrient-sensitive neurons have been shown to regulate the desire for food intake and satiety in response to peripheral energy expenditure. The localization of CPT1C to brain, a tissue normally not using fatty acids as a primary energy source, suggests a potentially unique function for CPT1C.

Cpt1c knock-out mice have been reported to have a reduced body weight on a normal diet, but when fed a diet that is rich in fats, these mice demonstrate an increase in relative body weight concurrent with a reduction in both food intake and insulin resistance. The expression of CPT1C in the hypothalamic feeding centers combined with the metabolic phenotype displayed by knock-out mouse suggest for a regulatory role of CPT1C in peripheral energy sensing and energy homeostasis.

In addition to the role of CPT1C in normal brain, CPT1C has also been implicated in carcinogenesis. It has been demonstrated that CPT1C expression is induced by hypoxia and p53-stabilization. It has been shown previously, that CPT1C expression is increased in lung tumor samples if compared to normal tissue. Furthermore, cells depleted from CPT1C display a significant reduction in cell proliferation in response to hypoxia.

The results presented here show that CPT1C expression is regulated by the presence of an upstream open reading frame (uORF) located the 5´UTR of the CPT1C mRNA. The presence of one or more uORFs is conserved in CPT1C mRNAs of different species but not within the CPT1 family of proteins. We showed that CPT1C expression is repressed by the presence of the uORF in the feeding state. This translational repression is released in response to reduced energy availability and AMPK activity. Our results furthermore show that the translation of the main ORF is also derepressed in response to Palmitate-BSA treatment. These results further support the hypothesis suggesting that CPT1C plays an important role in the nervous system control of energy homeostasis and peripheral energy expenditure although the involved signaling cascades still need to be established.

We observed that CPT1C expression was also induced in response to HIV infection. HIV has been shown to remodel the host cell mechanism to facilitate virus production, which leads to increased β-oxidation rates. Induction of CPT1C expression in response to virus infection might be used to remodel cell metabolism in order to facilitate the production of new viruses.

Furthermore, we were able to show that the induction of CPT1C in response to hypoxia was mediated by the Hypoxia-inducible factor (HIF) and that CPT1C expression correlates with the HIF target PGK in pediatric brain tumors. We showed that constitutive expression of CPT1C results in increased levels of cell proliferation and motility in established breast and brain cancer cell lines. Down regulation of CPT1C using shRNAs reduced cell proliferation and motility. In contrast to the metastatic phenotype that was observed under normoxic and hypoxic conditions, the reduction in cell proliferation was only in response to hypoxia. Cancer cell proliferation and metastasis in the hypoxic zone of solid tumors are mainly controlled by the HIF transcription factor complex. Induction of CPT1C expression in response to tumor hypoxia might present a survival mechanism for hypoxic tumor cells. Our

results add further evidence to the hypothesis that CPT1C contributes to metabolic adaptation of tumor cells in response to hypoxia. Activation of CPT1C in response to tumor hypoxia might lead to the activation of downstream pathways that serve to protect the cancer cells from metabolic stress. Further studies are necessary to establish the molecular function of CPT1C and the underlying signaling cascades.
Understanding the role of CPT1C in cancer cell metabolism might lead to the development of new approaches for the treatment hypoxic tumors.

Zusammenfassung

Die Carnitine Palmitoyltransferase 1C (CPT1C) wurde kürzlich als p53-induziertes, hirnspezifisches Mitglied der Carnitine Palmitoyltransferase (CPT) 1 Familie identifiziert. Die Mitglieder der CPT1 Familie regulieren den Import von Fettsäuren in die Mitochondrien und sind der limitierende Schritt der Fettsäureoxidierung.
Die physiologischen Funktionen der Leberisoform CPT1A und der Muskelisoform CPT1B sind aufgrund ihrer Beteiligung an der Entstehung von metabolischen Krankheiten gut beschrieben. Im Gegensatz dazu ist die Funktion der Hirnisoform bisher noch ungeklärt.
CPT1C konnte in den Neuronen des gesamten Hirnes nachgewiesen werden. Die höchste Konzentration findet sich aber in Hirnarealen welche mit der Regulierung der Nahrungsaufnahme und des Sättigungsgefühls assoziiert sind. Dazu gehören unter anderem der Hypothalamus, die Amygdala und der Hippocampus.
Die Expression von CPT1C im Gehirn, einem Gewebe welches normalerweise Fettsäuren nicht als primäre Energiequelle verwendet, deutet auf eine einzigartige Funktion für CPT1C hin. Frühere Studien zeigen das Cpt1c defiziente Mäuse ein reduziertes Körpergewicht haben wenn sie mit einer normalen Diät gefüttert werden. Werden diese Mäuse aber mit einer fettreichen Diät gefüttert, zeigen sie eine signifikante Gewichtzunahme in Kombination mit reduzierter Nahrungsaufnahme und Insulin Resistenz. Die Expression von CPT1C in Hirnarealen welche die Nahrungsaufnahme regulieren und der metabolische Phänotyp der Cpt1c defizienten Mäuse implizieren eine regulatorische Rolle innerhalb der Regulierung der Energie Homeostase für CPT1C.
Zusätzlich zur physiologischen Funktion im Gehirn, ist CPT1C auch mit der Krebsentstehung in Verbindung gebracht worden. Es wurde gezeigt, dass CPT1C Expression durch Hypoxie und p53 Stabilisierung induziert wird. Zusätzlich haben Studien gezeigt das CPT1C Expression in Lungentumoren im Vergleich zum Normalgeweben deutlich erhöht ist. CPT1C defiziente Zellen zeigen eine Reduktion der Zellteilung unter hypoxischen Bedingungen.
Die hier vorgestellten Resultate zeigen das die Expression von CPT1C durch die Anwesenheit eines upstream open reading frames (uORF) in der 5´UTR reguliert wird. Die Präsenz von einem oder mehreren uORFs konnte in den CPT1C mRNAs von verschiedenen Säugetieren nachgewiesen werde, jedoch nicht in den mRNAs der anderen CPT Isoformen. Unsere Resultate zeigen dass die Translation der CPT1C mRNA durch die Präsenz des uORFs inhibiert wird. Die Translation der CPT1C mRNA wird durch eine Reduzierung der Energiezufuhr und die Aktivität von AMPK aktiviert. Zusätzlich wird die translationelle Inhibierung auch durch die Zufuhr von Palmitat-BSA aufgehoben.
Diese Ergebnisse unterstützen die Hypothese, das CPT1C eine wichtige Rolle in der Regulierung der Energie Homeostase und des peripheren Energieverbrauchs durch das Nervensystem spielt. Die Signalkaskaden welche in diese Regulierung involviert sind, müssen durch weitere Studien etabliert werden.
Außerdem haben wir beobachtet dass die Expression von CPT1C durch die Infektion mit HIV induziert. Frühere Studien zeigen dass die Infektion mit HIV zu gravierenden Änderungen im Zell Metabolismus führt, welche die Produktion von neuen HIV Viren zu ermöglichen. Die Veränderungen im Zell Metabolismus führen zu einen Aufregulierung der Fettsäure Oxidation. Die Induktion der CPT1C Expression kann dabei einen Weg zur Umstellung des Metabolismus nach der HIV darstellen.
Die Expression von CPT1C wird außerdem durch die Stabilisierung von HIF induziert. In pädiatrischen Hirntumoren koreliiert die Expression von CPT1C mit der Expression von PGK eines HIF-induzierten Genes welcher als Marker für die Tumorhypoxie dient.
Die Überexpression von CPT1C in etablierten Brust und Hirn Tumor Zelllinien führt zu verstärkter Zellproliferation und Zellmobilität. Die künstliche Reduzierung von CPT1C durch shRNAs führt zu einer Reduction von Zellproliferation und Motilität. Im Gegensatz zum

metastatischen Phänotyp wurde die Reduktion der Zellproliferation unter hypoxischen Bedingungen beobachtet. Tumorzellproliferation und Metastasierung in den hypoxischen Zonen solider Tumore werden hauptsächlich durch den HIF-Transcriptionskomplex reguliert.
Die Hypoxie-induzierte Anreicherung von CPT1C könnte eine Möglichkeit darstellen das Überleben von hypoxischen Tumorzellen zu ermöglichen.
Unsere Ergebnisse unterstützen die Hypothese, dass CPT1C zur metabolischen Anpassung von hypoxischen Tumor Zellen beiträgt. Die Induktion der CPT1C Expression durch Tumor Hypoxie könnte zur Aktivierung von Signalwegen führen, die Tumor Zellen vor metabolischem Stress schützen.
Weitere Studien sind nötig, um die molekulare Funktion von CPT1C und die dem Phänotyp zugrundeliegenden Signalkaskaden etablieren.
Eine tiefere Einsicht in die Rolle von CPT1C im Tumorzellmetabolismus könnte zur Entwicklung von neuen Therapieansätzen für die Behandlung von hypoxischen Tumoren führen.

Table of Content

Abbreviations

µM	micro Mol
ACC	Acetyl-CoA-Carboxylase
AICAR	Aminoimidazol-Carboxamid-Ribonucleosid
AMP	Adenosinmonophosphate
AMPK	AMP-activated protein kinase
ASN	Asparagine
ATCC	American Type Culture Collection
ATG	start codon
ATP	Adenosine-5'-triphosphate
BM	basement membrane
BSA	bovine serum albumin
CACT	Carnitine Acylcarnitine Translocase
cDNA	complementary DNA
cGy	centi-gray
CNS	central nervous system
CoA	Coenzyme A
CPT1	Carnitine Palmitoyltransferase 1
CPT1A	Carnitine Palmitoyltransferase 1 liver isoform
CPT1B	Carnitine Palmitoyltransferase 1 muscle isoform
CPT1C	Carnitine Palmitoyltransferase 1 brain isoform
CPT2	Carnitine Palmitoyltransferase 2
CMV	Cytomegalovirus
CNS	Central Nervous System
DMEM	Dulbecco's Modified Eagle Medium
DMOG	dimethyloxaloylglycine
DNA	deoxyribonucleic acid
DPP	dose per pulse
DR	dose rate
DSB	double strand break
ECL	enhanced chemiluminescence reaction
ECM	extracellular matrix
EM	electron microscopy
FA	fatty acid
FAD	flavin adenine dinucleotide
FAO	fatty acid oxidation
FAS	Fatty Acid Synthetase
FBS	fetal bovine serum
FFA	free fatty acid
FFF	flattening filter free
GAPDH	Glycerinaldehyd-3-phosphat-Dehydrogenase
GBM	glioblastoma multiforme
GFP	green fluorescent protein
Gy	gray
H&E stain	hematoxylin and eosin stain
HDAC	Histone Deacetylases
HFD	high fat diet
HIF	Hypoxia-inducible factor
HIV	human immunodeficiency virus
HNPCC	hereditary nonpolyposis colorectal cancer

HRP	horse raddish peroxidase
I.P.	intraperitoneal injection
IR	ionizing radiation
LCFA	long chain fatty acid
LMNA	Lamin A
LTR	long-terminal repeat
MCD	Malonyl-Decarboxylase
Min	minute
mORF	main ORF
M	mol
mRNA	messanger RNA
MU	monitor units
NAD^+	nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ORF	open reading frame
PCNA	Proliferating Cell Nuclear Antigen
PCR	polymerase chain reaction
PGK	Phosphoglycerate Kinase
PHD	Prolyl-4-Hydroxylase
PPAR	peroxisome proliferatoractivated receptors
PRF	pulse repetition frequency
PRO	prolin
PVDF	polyvinylidene difluoride
qRTPCR	quantitative real time PCR
RNA	ribonucleic acid
RLU	relative luciferase units
S.C.	subcutaneous injection
SCFA	short chain fatty acid
SD	standart deviation
SDS	sodium dodecyl sulfate
shRNA	short hairpin RNA
shRNAmir	microRNA-adapted shRNA
SREBP	sterol regulatory element-binding protein
uATG	upstream ATG
uORF	upstream ORF
UTR	untranslated region
VEGF	Vascular Endothelial Growth Factor
VHL	von Hipple-Lindau
VSV	vesiculo-stomatitis virus
WHO	World Health Organization
wt	wild type

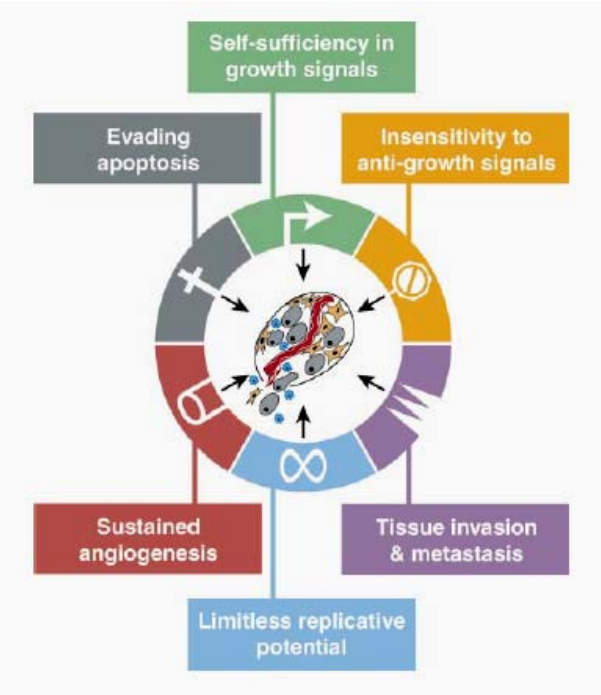
1 Introduction

1.1 Cancer

Cancer is, after cardiovascular diseases, the second leading cause of death world wide (WHO).
In Switzerland over one quarter of all death cases during the last decade were caused by cancer (WHO). In Europe Lung cancer is the leading cause of cancer death in adults while leukemia is the most common cancer in children (WHO). The incidence of particular cancers varies around the world and sometimes according to the ethnic group. Over the next decades the incidence of various cancer types will dramatically increase due to an aging population and the maintenance of an unhealthy lifestyle. While it is well established that main risk factors for cancer development are genetic predisposition and the exposure to cigarette smoke, alcohol abuse and environmental toxins like asbestos and pesticides, the impact of wrong eating habits and obesity still need to be fully established. Risks to humans from carcinogens depend upon the dose and susceptibility. Factors influencing the susceptibility to cancer include age, sex, nutritional status and genetics. All cancers result from mutations of genes that allow the cells to begin an uncontrolled growth. These mutations can be either inherited or acquired. Only 5% of all cancers in Europe are thought to be explained by inherited genetic mutations. Known genes associated with hereditary cancer include the mutated BRCA1 and BRCA2 genes that increase breast cancer risk (50) and the HNPCC gene that is linked to colon cancer (221). Acquired mutations are caused by repeated exposure to carcinogens. The most significant avoidable carcinogens are chemical components like asbestos, pesticides or tobacco smoke. Dietary components, like excessive consumption of alcohol or of foods high in fat have also been linked with various cancers. These cancers also account for most of the cancer-related deaths due to the high amount of occurrence and malignancy.
Additionally, infections with various pathogens are also known to cause cancer. The bacterium *Helicobacter pyloris*, for example, is known to cause ulcers and thereby increase the risk of stomach cancer (National Cancer Institute). Furthermore, several viruses, like members of the herpes virus group, human papilloma viruses and the human immunodeficiency virus, have been shown to be involved in the development of numerous cancers.
There is usually a latency period of years or decades between exposure to a carcinogen and the appearance of cancer. This, combined with the individual nature of susceptibility to cancer, makes it very difficult to establish a cause for many cancers (168, 225).

1.1.1 Tumor development

Most DNA damages induced by carcinogens come to nothing because of the cellular DNA damage repair pathways that in normal cell repair any occurring DNA damage. Repeated exposure to DNA-damaging agents however can result in mutations or altered gene expression in so-called oncogenes and tumor-suppressor genes. Oncogenes mostly produce growth factors that activate signaling cascade that allow a cell proceed through the cell cycle and finally divide. Tumor suppressor genes on the other hand encode for factors that lead to the activation of signaling cascades that regulate for example cell cycle checkpoints, DNA damage repair or apoptosis. While mutations in oncogenes can lead to an activation of growth signaling cascades resulting in abnormal cell proliferation without exogenous signals, mutation in tumor suppressor genes allow the proliferation of damaged cells and thereby leading to further acquisition of mutations, neoplastic transformation and finally tumor formation. Although the signaling cascades involved in the neoplastic transformation of tumors cells can vary in different tissues, the underlying concept remains the same.



In many cases the tumor remains small and localized for years, but after some time eventually begins to grow and spread.
It is well established that tumor cells have to acquire the same six hallmarks to establish a tumor (Fig. 1). The order, in which these hallmarks are acquired, however, can vary significantly between different tumor types (32). Furthermore, the mutations needed in order to acquire the hallmarks can not only vary between tumor types but also between individuals having tumors of the same type.
One of these hallmarks is the self-sufficiency in growth signals. Cells within healthy tissues only divide if they receive growth signal from other cells. These signal are detected by growth factor receptors such as the vascular endothelial growth factor

Figure 1: The Hallmarks of cancer.
This model defines the six common properties that tumors acquire to survive and metastasize.
Adapted from Hanahan & Weinberg (2000) The Hallmarks of cancer. *Cell* 100: 57-70

receptor. In response to ligand binding these receptors activate signal cascades stimulating cell proliferation and inhibiting apoptosis. Tumor cells in contrast do not depend on the stimulation of cell proliferation by exogenous growth signals. This can be achieved by a number of different approaches. Tumor cells can reduce the dependency on exogenous growth factors on the one hand by the endogenous production of growth signals and on the other hand by the modification of the total amount and activity of the growth factor receptors. Mutations that render the receptors constitutively active, leads to the activation of signal transduction pathways even in the absence of mitogenic factors. Additionally, tumor cells show modifications of the cytoplasmic components that regulate cell proliferation. Mutations of intracellular factors such as Ras, which is mutated in around 20% of all tumors, lead to the permanent stimulation of proliferation.

1.1.2 Tumor hypoxia

All solid tumors independent of their origin contain areas of low oxygen tension. Solid tumor growth is limited by the tumors potential to induce the intratumoral development of blood vessels. Without tumor vascularization tumor size is limited to a diameter of around 2mm. Tumor hypoxia occurs at a distance of 100-200µm from the blood vessel. Cells that are not able to adapt to the hypoxic conditions and survive the oxygen and nutrition deprivation will undergo apoptosis.
Normal cells and tumors need oxygen to generate energy and to enforce most of the fundamental processes driving cell proliferation and survival. In tumor cells nevertheless, hypoxia is also strongly associated with tumor progression and metastasis (36, 91, 119, 121, 125, 130, 144, 161).
It is well established that tumor hypoxia induces an epigenetic selection for cancer cells that survive in the hostile tumor environment (119, 121, 125, 130, 159, 218). Emerging evidence suggests that the effect of hypoxia is controlled by Hypoxia-inducible factor (HIF) -mediated

activation of pathways which enable tumor cells to survive or escape their oxygen-deficient environment (91, 119, 121, 125, 130, 144, 159, 170).
Under normoxic conditions HIF1α is unstable and rapidly degraded after protein translation. HIF1α stability is regulated by two prolines in the HIF1α oxygen-dependent degradation domain that are hydroxylated under normoxic conditions by a family of oxygen-dependent prolin hydroxylases (PH) and subsequently ubiquitinylated by the von Hipple-Lindau tumor suppressor. In response to hypoxia HIF1α is stabilized and associates with the ubiquitously expressed β-subunit. Binding of transcriptional co-activators such as p300 activates the transcription of a growing number of genes containing hypoxia-responsive elements (Fig. 2). HIF induces the transcription of a growing number of target genes that mediate a wide range of cellular processes such as angiogenesis, glycolysis, cell proliferation, cell survival and cell death (65, 69, 88, 91, 119, 121, 122, 125, 127, 130, 170).
It has been suggested by earlier publications that HIF plays an important role in carcinogenesis. This is supported by the fact that HIF is activated in the majority of human cancers, where it is mainly expressed in the hypoxic zones. Additionally, most HIF target genes found in hypoxic tumors are strongly associated with cancer progression and metastasis. In tumor cells HIF promotes the selection and expansion of more aggressive clones with a reduced apoptosis potential. This may explain why hypoxic tumor cells are mostly resistant to therapeutic approaches. In tumor cells HIF influences the tumor cell phenotype by activating key regulators of angiogenesis (91, 127), metastasis (91, 121, 144, 161) and energy metabolism (86, 91, 121, 125, 130, 159, 225).

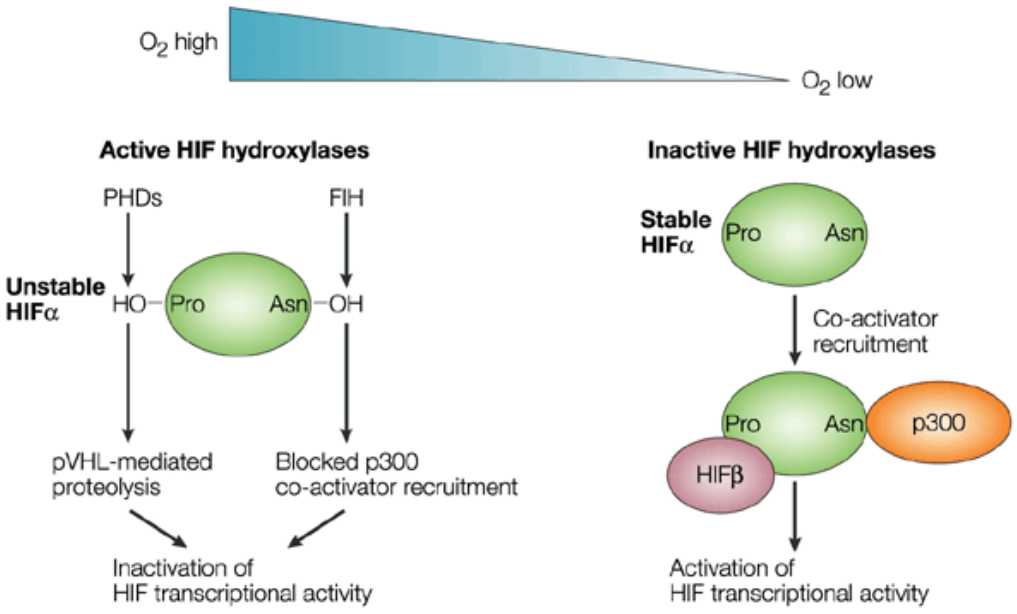


Figure 2: Regulation of HIF stability by cellular oxygen availability.
Under aerobic conditions, HIFα is hydroxylated on proline 402 and proline 564. The proline hydroxylations are necessary for binding to von Hippel-Lindau (VHL) and ubiquitin-mediated degradation by the proteasome. The asparagine hydroxylation prevents binding to p300. Under hypoxic condition, HIFα is stabilized binds to HIFβ and p300 and transactivates the transcription of hypoxia responsive genes.
Adapted from: Schofield et al. (2004) Oxygen sensing by HIF hydroxylases. *Nature Reviews Molecular Cell Biology* 5: 343-354

Pharmacological inhibitors that block HIF signaling or HIF target genes such as VEGF have been proven to be successful cancer therapeutica (105). The identification of novel HIF target genes involved in tumor progression will lead to a better understanding of the molecular pathways involved in HIF-promoted selection of aggressive cancer cells and might thereby suggest new approaches for cancer therapy.

1.1.3 Metabolic adaptation of tumor cells

Cancer cells have different metabolic demands than normal cells to meet the requirements of rapid cell proliferation as well as autonomous growth and survival in an environment absent of contact with extracellular matrix. It is well established by a number of studies that the metabolic adaptation is required to support the proliferation of cancer cells. During carcinogenesis, cells acquire mutations that confer selective advantages for the growth of the tumor. It is hypothesized that most of the known oncogenes and some tumor suppressor genes regulate pathways and downstream target genes that are connected to the metabolic regulation.
In most tumor types the rate of glucose uptake is increased while the rate of oxidative phosphorylation in mitochondria is reduced if compared to normal tissue. This effect is called aerobic glycolysis or Warburg effect (120, 130, 159, 209). It is well established that HIF activates angiogenesis to improve oxygen and nutrient supply in hypoxic tumors. Additionally the switch from oxidative phosphorylation to anaerobic glycolysis is a key player in the adaptive response to tumor hypoxia. HIF1 is the major transcription factor for the induction of most of the genes encoding glucose transporters and glycolytic enzymes (130, 159, 209) under hypoxic conditions.
Glucose is transported into the cell by glucose transporters, which compromise six isoforms that differ in tissue specificity and kinetic behavior regulating the tissue capacity of glucose uptake and utilization. Following glucose uptake, tumor cells convert glucose to pyruvate by aerobic glycolysis. Pyruvate is finally converted into lactate instead of undergoing oxidative phosphorylation through the citric acid cycle. Lactate dehydrogenase, a direct target of HIF1, is the enzyme responsible for the conversation of pyruvate to lactate by facilitating the glycolytic flux and maintaining the $NADH/NAD^+$ ratio under conditions of low oxygen. Earlier studies have demonstrated that HIF activation in hypoxic tumor cells correlates with increased glycolysis rates resulting in restored ATP levels and thus tumor growth (120, 125, 130, 159, 209, 218).
Although the metabolic adaptation of tumors is believed to mainly function through the glycolytic pathway, increasing evidence suggests the involvement of fatty acid oxidation (FAO) in this process (89, 102, 218, 222).
It has been reported that some fatty acids play a role in enhancing cell proliferation and represent an important energy source in slow growing hepatomas (232) and prostate cancer (102). On the other hand studies done in colon cancer cells revealed that an increase in carnitine-dependent fatty acid uptake into mitochondria induces apoptosis.
These studies show that the metabolic adaptation of tumors cells is not limited to changes in glycolysis and oxidative phosphorylation. Further studies are needed to elucidate the impact of FAO in the metabolic adaptation in cancers of different origins.

1.1.4 Metastasis

Metastasis represents the final and fatal stage of cancer and is the leading cause for cancer related death. In spite of the significant improvement of cancer prevention and early detection, a high number of tumors already gave rise to distant metastasis or are in the process of spreading at the time of diagnosis (WHO).

The process of cancer cell invasion and metastasis and the molecular mechanism underlying this process, although accounting for 90% of cancer deaths (WHO), are still insufficiently understood.
Once the primary tumor has formed up to 1 million cells can leave the tumor mass per day and enter the metastasis cascade. Fortunately the metastatic process is not very efficient. Due to that the amount of metastasis which evolves into a clinical disease is relatively low. Furthermore it has been demonstrated by clinical studies that the metastatic potential not only depends on tumor type and grading but also on the immunological status of the patient.
Tumor invasion and metastasis through the bloodstream to an anatomically distant side is a coordinate multistep process that involves the activation of complex signaling cascades (54, 100, 173, 187, 190, 201, 203, 217).
The metastatic cascade can be dived into four major steps: (1) the release of tumor cells from the primary tumor mass, (2) invasion of the basement membrane (BM) and migration through the extracellular matrix (ECM) surrounding the tumor mass and subsequent invasion of the basement membrane of the endothelium in order to allow intravasation, (3) adhesion to the endothelial cells and extravasation, (4) and finally colonization of the tissue and tumor cell proliferation (Fig. 3). Prior to the actual activation of the metastatic cascade, the tumor has to activate angiogenesis to allow the development of new vessels to increase the blood supply of the growing tumor. Without the formation of intratumoral vessels a tumor would not be able to grow beyond 2mm of diameter and in addition it is well established that the metastatic cascade can not be activated without the formation of blood supply to the primary tumor.
Cancer cells must become motile in order to leave the primary tumor and move to the metastatic side. The first step of this process is the disaggregation of tumor cells from the primary tumor mass which results from a loss of cell-cell-adhesion mediated by altered expression of adhesion molecules like cadherins and catenins (51, 100, 103, 145, 152, 173, 181, 187, 190, 217).

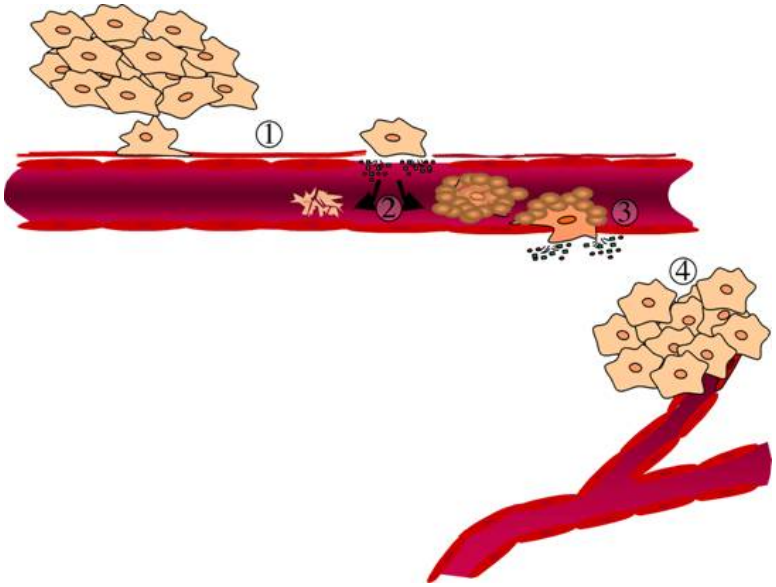


Figure 3: The metastatic cascade.
(1) De-adhesion of tumor cells from the primary tumor, (2) migration through the ECM and intravasation, (3) adhesion of tumor cells to the endothelial cells and extravasation, (4) proliferation of tumor cells and establishment of a distant metastasis
Adapted from Gomez et al. (2004) Potential application of desmopressin as a preoperative adjuvant in cancer surgery. Biological effects, antitumor properties and clinical usefulness. *Cancer Therapy* 2: 279-290

After overcoming the adhesive constraints, the cells have to invade and migrate through the BM and ECM to intravasate the vessel. This is facilitated by the expression of proteolytic enzymes such as Matrix-Metalloproteases that degrade matrix proteins and thus allow the invasion through the stroma. Upon physical contact between tumor and endothelial cells mediated by integrins, the endothelial cells retract and allow the tumor cells to enter the blood

circulation. Once in the circulating system the tumor cells have to adapt to the shearing forces generated by the blood flow and evade attacks from the immune system. When the tumor cells reach the capillary bed of the target organ they attach to the endothelial cells, stimulate endothelial retraction and invade the target organ.
A critical step in the formation of distant metastasis is the adaption of tumor cells to the new microenvironment after extravasation. Tumor cells might depending on the primary tumor type and aggressiveness either undergo apoptosis, remain dormant or start proliferating immediately. Only a very small portion of the cells that enter the circulating system will succeed in forming a metastasis in the secondary tissue.
Tumor cell invasion is widely influenced by the tumor microenvironment. Hypoxic conditions within the primary tumor mass activate signaling cascades that stimulate cancer cells spreading by altering gene expression through hypoxia-responsive proteins like HIF1. HIF1 transcriptionally activates genes involved in the activation of the metastatic cascade (91, 121, 125, 144, 161, 187) in order to ensure cell survival (227). The molecular mechanism by which tumor hypoxia might induce tumor cell migration are not fully elucidated yet. Nevertheless, increased mutation rates, genomic instability and a strong epigenetic selection for cancer cells that survive the hostile tumor environment are likely to be involved in this process (144, 181, 190, 219).

1.1.5 Breast cancer

Breast cancers can occur in men and women but are mostly found in women, where they are the most prevalent cancer as well as the leading cause for cancer death.
Breast cancers originate from breast tissue, most commonly from the milk ducts or the lobules that supply the ducts with milk. There are many different types of breast cancers, with different stages and aggressiveness. Breast cancers can spread to almost any area of the body. The most common regions that breast cancers may spread to are bone (25%), lung (21%), liver (15%) and brain (5%) (87, 205).
Patient survival varies from 98% to 10% depending on tumor stage and treatment. Due to today's prevention strategies leading to earlier tumor detection and the improvement of breast cancer therapy, the survival rates have markedly improved. Metastasis occurring in the later stages nevertheless represents the most serious thread for patient survival (205).

1.1.6 Brain cancer

There are two types of brain tumors: primary brain tumors that originate in the brain and secondary brain tumors that originate from cancer cells that have migrated from other parts of the body.
Primary brain tumors are divided into nine categories, which are based on the types of cells in which the tumors originate. Gliomas are primary brain tumors that are made up of glial cells which provide important structural support for the nerve cells in the brain. All gliomas range from well-differentiated low grade tumors to anaplastic high grade tumors. Anaplastic tumors independent of the cellular origin are completely undifferentiated, chaotic tumors that are highly aggressive and associated with low survival rates (26, 61, 68, 192).
Primary brain tumors although rarely spreading beyond the central nervous system (CNS), spread within the brain tissue and death is induced by tumor growth within the limited space of the skull.
Secondary brain cancers occur in 20–30% of patients with metastatic disease and the incidence increases with age. Generally brain cancer metastasis occurs only in the advanced disease and has a poor prognosis (150, 213).

Glioblastoma multiformae, which represent 23% of all brain tumors, are among the most malignant human cancers with a five-year survival rate of 4.9% in patients older than 50 years. Following a short period of remission, the tumors almost always recur and the patient succumbs to the fast progressing disease.
Though intensive research has been done on brain cancers over recent years, the prognosis for brain cancer patients has hardly improved. Histological tumor staging and patient therapy are still limited due to missing molecular markers that would allow a prediction of tumor aggressiveness and its sensitivity to therapy (181, 202, 204, 212).

1.1.7 Cancer treatment

Cancer therapy is mainly based on three treatment modalities: surgery, chemotherapy and radiotherapy. Apart from that, new cancer treatments like antibody- and hormone-based approaches are continuously developed and tested in clinics (105, 167, 194).
If allowed by tumor type, location and patient health, the tumor and surrounding infiltrated tissue is removed by surgery. Either in addition to surgery or as a replacement, most tumors are treated with radio- and/or chemotherapy.
Chemotherapy provides a systemic approach for cancer therapy. Most therapeutic drugs poison cells by either impairing cell division or metabolism and thereby effectively kill fast-dividing cells. Because chemotherapy mainly affects cell division, tumors with high growth fractions such as aggressive lymphomas are more sensitive to chemotherapeutic drugs than slow growing tumors such as slow growing lymphomas (40, 77).
Due to its systemic administration, chemotherapy not only impacts on tumor cells but also affects fast dividing healthy cells like hematopoietic cells, hair follicles and intestinal cells.
Further studies are necessary to identify new drug targets that allow the specific elimination of tumor cells and yet display low side effects.
Radiotherapy represents one of the most effective tools in the treatment of human cancers. In contrast to chemotherapy, radiotherapy allows the local application of the ionizing radiation (IR) and thereby avoiding the systemic side effects induced by chemotherapy. Linear accelerators used for patient treatment allow the fast and precise irradiation of tumors located in virtually any part of the human body (188).
It is well established that cells exposed to IR show DNA lesions, increase mutation rates, chromosome aberrations and finally apoptosis. DNA damage induced by IR arises not only from direct effects but is also induced by reactive oxygen species derived from water hydrolysis. Although IR induces a number of different DNA lesions, DNA double strand breaks (DSB) are the most damaging DNA lesion because of their impact on genome stability and proliferation (1, 31, 74, 92, 193, 208). Once detected DNA damages are repaired by specific DNA damage repair pathways that utilize different set of proteins depending on the induced DNA lesion. Furthermore, signaling cascades that slow or arrest the cell cycle are induced to delay cell cycle progression until the damage is repaired. If the lesion can not be repaired, pro-apoptotic pathways are induced to remove the damaged cells from the proliferative pool.
Although today's cancer therapies allow the efficient elimination of tumor cells, some tumor types for example glioblastomas are still mainly resistant to therapy. The same is true for hypoxic tumors which represent a serious tread to patient survival because of the high rate of surviving tumor cells and the ability of tumor hypoxia to induce pathways leading to metastasis (40, 71, 77, 127, 193, 196, 202). New approaches are needed to render hypoxic cells sensitive to cancer therapy.

1.2 Regulation of gene expression

Physiological processes important in cell growth and metabolism as well as stress-induced pathways depend on the regulation of gene expression. This regulation can occur on various levels from the regulation of gene transcription to the regulation of protein stability (Fig. 4).
The regulation of transcription, which is working through a canopy of transcription factors, is well studied and understood (12, 30, 47, 233, 234). A determination of the mRNA level, however, is not predictive for the level of the encoded protein (35). Gene expression is further regulated at the step of RNA processing and translational initiation to allow the modulation of translation efficiency and RNA stability (3, 12, 14, 30, 37, 152). It is important that this multi-step process is tightly controlled to ensure that all steps function in a concerted manner. Specific signal cascades are used to regulate each step of gene expression to integrate cellular and environmental signals.

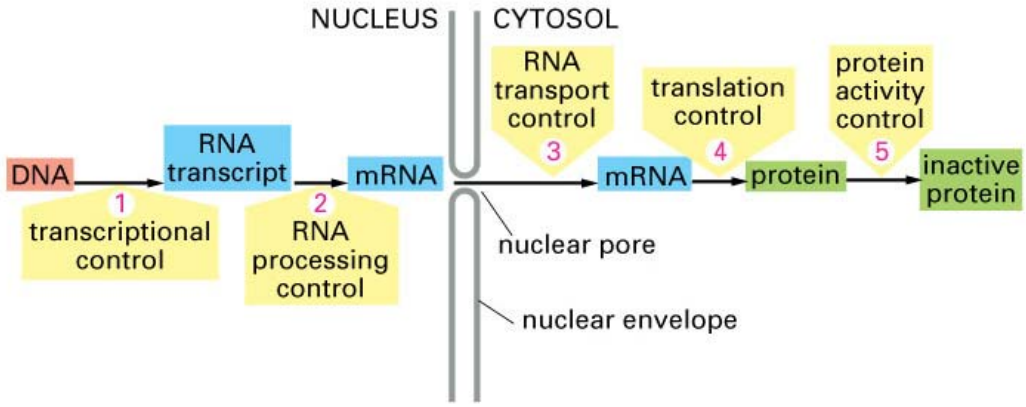


Figure 4: The regulation of gene expression.
Adapted from: Essential Cell Biology, Second Edition**,** Garland Publishing

1.2.1 Transcriptional regulation of gene expression

The transcriptional regulation of gene expression controls which gene is transcribed, when transcription occurs and how much of a certain mRNA is transcribed. mRNA transcription is regulated by a number of mechanisms such as the regulation of histone remodelling, the regulation of transcription factor expression and stability, and the use of transcriptional enhancers and repressors (233, 234).
Histon proteins assemble with the DNA to form nucleosomes which consists, the basic repeating unit of chromatin. The nucleosome contains 146bp of DNA wrapped around a protein core consisting of each two copies of the four histone proteins H2A, H2B, H3A and H4. Untranscribed regions are packed into highly condensed heterochromatin while transcribed genes are packed into the more accessible euchromatin. The DNA of each cell type is packed into a unique pattern of hetero- and euchromatin determining the unique characteristics of each cell line. This pattern is maintained after cell division to transfer the lineage specific gene expression pattern to the next generation.
To activate gene expression, transcriptional activators need to bind and remodel the chromatin structure to allow DNA accessibility.

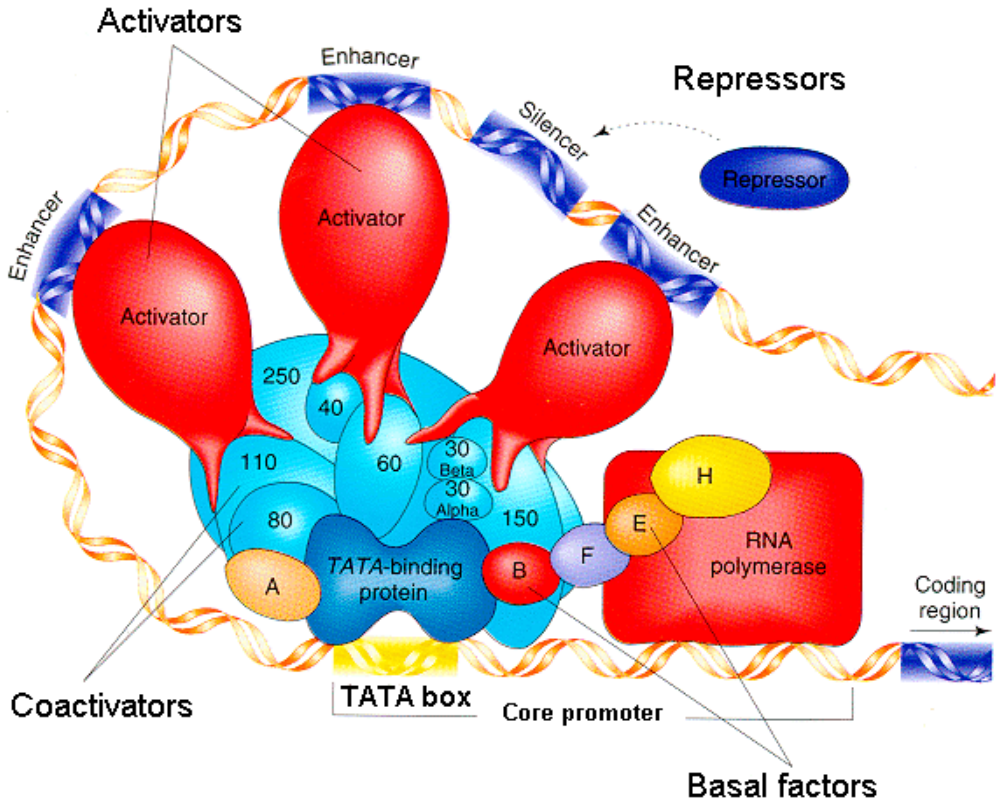


Figure 5: The molecular apparatus controlling transcription.
Four kinds of components control transcription in eukaryotic cells. Basal transcription factors bound to the TATA box-binding protein are the minimal requirement for RNA polymerase II recruitment and transcriptional initiation. Activators and repressors are regulatory sequence-specific DNA-binding proteins that communicate with the basal transcription factors via co-activators and act to regulate transcription efficiency.
Adapted from Tjian R. (1995) Molecular Machines That Control Genes**.** *Scientific American* 272 (2): 54-61

The transcription of most human genes is regulated by transcription factors that bind specific DNA regulatory sequences upstream of the core promotor. The activity of most transcription factors is controlled by regulatory signaling pathways that modulate transcription in response to diverse endogenous or exogenous signals. Transcription factors can either act as transcriptional enhancers or repressors. The action of a certain transcription factor can vary depending on the transcribed gene and cell type. The transcription factors bind to the regulatory elements and promote access to the DNA through interactions with other components of the transcription machinery. The transcription is initiated by the recruitment of the RNA polymerase II to the transcriptional start site by the basal factors (Fig. 5). After transcriptional initiation, a cap structure is added 5´to the newly synthesized transcript which serves as a binding site for proteins involved in mRNA export to the cytoplasm and translation. During transcription elongation, which is regulated by elongation factors, the polymerase moves 5´to 3´along the gene sequence and extends the transcript. During the elongation of the synthesized mRNA, non-coding sequences (introns) are removed by pre-mRNA splicing. At the end of the gene, the transcription is terminated and the synthesized mRNA is cleaved and the PolyA tail is added 3´to the end of the transcript. After transcription and transcript processing, the mRNA is transported to the cytoplasm for translation.

The transcription of genes is influenced by the cell environment, metabolic status and cell lineage through the differential expression and activity of a multitude of transcription factors (88, 151, 229, 233).
In addition to the role if fatty acids (FAs) as an energy source, FAs have been shown to affect gene expression of genes involved in the regulation cell proliferation, differentiation, metabolism and apoptosis. FAs regulate the transcription of target genes such as CPT1 and Acyl-CoA Synthetase via changes in activity or abundance of several transcription factors like PPAR, SREBP and NFκB. The FA control of these transcription factors involves the direct binding of FA to the transcription factors or the indirect activation of transcription factors by the activation of FA-induced signaling cascades (9, 24, 41, 67, 214). Epigenetic changes induced by dietary FAs have been implicated in a number of diseases like coronary artery disease, insulin resistance, hypertension and cancer (24, 97, 109, 134, 177, 178). In cancer, FAs have been hypothesized to alter the expression of genes involved in cell growth, metabolism and adhesion and thereby contribute to cancer progression (102, 218, 220, 222).
As mentioned earlier, hypoxia has profound influence on cellular gene expression. The main transcription factor involved in the hypoxia-dependent transcriptome remodeling is HIF (65, 81, 88, 229). HIFα is stabilized in response to hypoxia and leads to the transcription of target genes containing a hypoxia response element after polymerization with HIFβ and p300 (Fig. 2). In addition, the HIF-binding protein p300 is a histone acetyl-transferase that plays an important role in the full activation of HIF-target gene transcription by modifying the chromatin structure (68, 81, 125). Interestingly, histone deacetylases (HDAC) have also been shown to be interaction partner of HIF (81, 125) and are associated with HIF transcriptional activity. Binding of HDACs to the HIFα leads to protein stabilization by binding to the oxygen-dependent degradation domain (81, 122, 133). Activation of HIF-dependent transcription allows cell survival under hypoxic conditions (81, 91, 121, 130).
Viruses are known to re-program cellular pathways to facilitate virus production. HIV infection of T lymphocytes leads to major changes in the transcriptome. It has been shown that HIV infection alters the expression of a number of transcription factors and genes involved in RNA processing in order to facilitate high HIV gene expression and virus packing (188). Changes in transcription factor expression and activity upon HIV infection have been shown to alter host cell metabolism by upregulation of β-oxidation and down regulating glycolysis (168). Furthermore, some viruses have been shown to induce the oncogenic transformation of the infected cell as a side effect of cell programming (168, 188) and thereby induce carcinogenesis.

1.2.2 Post-transcriptional regulation of gene expression

Translational control as a tool to regulate gene expression has been found in many species (35, 36, 57, 59, 139, 181). In most species, posttranscriptional regulatory mechanisms have been shown to play an important role in stress response pathways (47, 57, 78, 98, 110, 149, 160, 182, 206) and lead to dysfunctional regulatory pathways if blocked by mutations (20, 46, 104, 111, 132, 149, 153, 182).
The posttranscriptional events compromise pre-mRNA processing, mRNA localization, stabilization (3, 37, 166) and translation (8, 35, 152). Each event is tightly regulated due to the impact that each step can have on mRNA translation. The mechanisms regulating each step, although poorly understood, involve mRNA structural components and mRNA binding proteins (51).
Many of the mutations found in human cancers lead to truncated ORFs which reduces mRNA stability by the activation of the non-sense-mediated decay. Often mutations also affect mRNA splicing and generate alternatively splice mRNA which contain premature stop codons. Premature stop codons reduce mRNA stability by the induction of non-sense-

mediated decay. Non-sense-mediated decay is induced by slice site markers that remain bound to the mRNA after the first round of mRNA translation (35, 166, 182). This effectively prevents the production of truncated protein.
Post-transcriptional regulation of gene expression can also be controlled by short sequence elements located in the UTR´s of mRNAs. One of these elements found within the 5´UTR are upstream open reading frames (uORFs).
The presence uORF within the 5´UTR is crucial for the translation initiation of the main open reading frame (mORF) (38, 158, 182). uORFs are defined by a start codon that is out of frame of the mORF and a length of at least 9nt. It has been shown that mRNAs can contain one or more uORFs that are either fully upstream or overlapping with the mORF (Fig. 6). The uORF is except for a few cases (182) not encoding for a protein.
Because eukaryotic ribosomes generally only initiate once per mRNA (8), the presence of an uORF normally inhibits the translation of the mORF and may lead to mRNA decay (37, 153, 182). Earlier studies showed that ribosomes loaded on the 5´cap of uORF containing mRNAs can either only translate the uORF or reinitiate and also translate the mORF (182). It has been shown that many stress-induced genes contain uORFs. In these mRNAs the uORF is translated under normal conditions, while if the cells are exposed to the specific cell stress the ribosomes either reinitiate after the uORF or simply scan through the uORF leading to the translation of the mORF (182). In all genes where uORF have been identified so far the mere presence of the uORF is sufficient for the regulation of the mRNA translation and mRNA stability.
It has been estimated that approximately half of human protein-encoding genes contain uORFs (182), nevertheless, the impact of this post-transcriptional regulation mechanism on cellular signaling cascades is yet unsolved. Furthermore, the impact of RNA-binding proteins in this regulation needs further examination.
Although they are usually short sequences, uORFs may still play critical roles in modulating physiology. Indeed, mutations that introduce new or disrupt existing uORFs have been reported to cause human diseases (20, 46, 104, 111, 182). Additionally, recent publications also implicate abnormalities in post-transcriptional regulation pathways in cancer development (28, 104, 132, 153).

Figure 6: Schematic representation of a mRNA containing 2 uORFs in the 5´UTR. Adapted from Calvo et al. (2009) Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *PNAS* 106, 7507-7512

1.3 Hypothalamic regulation of peripheral energy homeostasis

The maintenance of whole body energy homeostasis is critical for survival. This requires the presence of sensors that detect changes in whole body energy expenditure and induce adaptive responses. Nutrient-sensitive neurons are found in the brain stem and hypothalamus (52, 63, 82, 93, 107). These regions play an important role in the nervous system control of energy homeostasis. Hypothalamic feeding centers have been shown to regulate the desire for food intake and satiety (29, 49, 52, 63, 82, 93, 107, 108, 117, 165).

The AMP-activated protein kinase (AMPK) has been shown to play an important role in the hypothalamic regulation of energy homeostasis. AMPK is a heterotrimeric kinase complex composed of a catalytic α subunit and two regularly β and γ subunits. In mammals, several alternative genes for each subunit have been identified that display a cell and tissue type specific expression pattern. AMPK activity is enhanced by AMP binding and phosphorylation of the catalytic subunit by upstream kinases.
Hypothalamic AMPK is responsive to alteration in cellular energy level, circulating hormones and nutritional cues (29, 44, 52, 63, 93, 117, 165, 224). The modulation of AMPK activity in response to these factors initiates signaling pathways leading to changes in feeding behavior (140, 165).
Hypothalamic AMPK activity is tightly regulated under physiological conditions. Different studies showed that injection of mice and rats with leptin decreased AMPK activity and lead to a subsequent reduction in feeding behavior (44, 52, 63, 82, 97, 117, 140). This was also true for other anorexic stimuli such as insulin, glucose and refeeding after a fasting period (44, 52, 117). These results show that a reduction in hypothalamic activity is sufficient to reduce food intake and body weight (44, 52, 97, 146). In contrast to these results, injection of the pharmacological AMPK agonist AICAR or the expression of constitutively active AMPK cause an increase in food intake and body weight (44, 82, 97, 146).
The molecular mechanism(s) by which AMPK regulates nutritional satiety involving the hypothalamus are still largely unknown but it may function through controlling differential hypothalamus gene expression.
In addition to the AMPK-mediated regulation of energy homeostasis, FAs are also involved in the nervous control of energy homeostasis (49, 52, 56, 72, 82, 97, 118, 131, 134, 178). It has been shown that free FAs are not used as fuel for neurons but serve as informative molecules about the whole body energy homeostasis (49, 72, 82, 97, 113, 134). Specialized neurons within the hypothalamus detect variations of FA levels in the plasma and integrate this information in the regulation of peripheral glucose and lipid metabolism. Inhibition of the hypothalamic Carnitine Palmitoyltransferase 1 (CPT1), the enzyme that controls the entry of long-chain-fatty acids (LCFAs) into the mitochondria for β-oxidation, by chemical inhibitors or the accumulation of LCFAs in the hypothalamus causes a reduction of food intake (49, 52, 56, 97, 107, 108, 118). It has been hypothesized that high FA levels might serve as a signal for sufficient feeding and thereby reduce food intake. Further studies are needed to elucidate the mechanisms by which CPT1 inhibition regulates feeding behavior and to elucidate the neurotransmitters used by these neurons.

1.4 β-Oxidation

LCFAs serve as an energy source for many tissues, especially heart and skeletal muscle. But some tissues like the brain, erythrocytes and adrenal medulla are not believed to utilize fatty acids for energy requirements.
β-oxidation is the process by which fatty acids are broken down in mitochondria and/or peroxisomes to generate Acetyl-CoA, the entry molecule for the Citric Acid cycle.
Fatty acid oxidation involves three specific steps: (1) uptake and activation of FFAs, (2) translocation of fatty acyl-CoA into the mitochondria for (3) β-Oxidation.
After their entry into the cell through specific membrane transporters, FAs must be esterified to Coenzyme A (CoA) before they can undergo oxidative degradation. Acyl-CoA Synthetases located in the outer mitochondrial membrane catalyze the activation of LCFAs by esterifying them to Coenzyme A. In order to enter the mitochondria for β-Oxidation, the FAs are conjugated to carnitine. This is facilitated by CPT1, which is located in the outer mitochondrial membrane with the active center facing the cytoplasm (17, 53, 210). The acyl-

carnitine can freely migrate through the outer mitochondrial membrane and is then translocated through the inner mitochondrial membrane by the Carnitine Acylcarnitine Translocase (CACT) which exchanges one molecule long-chain acylcarnitine for one molecule of carnitine. Once inside the mitochondria the fatty acid is conjugated back to CoA while carnitine is released to the mitochondrial matrix. The generated fatty acyl-CoA enters β-Oxidation (Fig. 7).

Each cycle of β-Oxidation occurs in a sequence of four reactions that reduce the FA chain length by a two carbon unit-acetyl CoA. The first step of β-Oxidation is the dehydrogenation of the fatty acid by the Acyl-CoA-Dehydrogenase which catalyses the formation of a double bond between the C2 and C3. In the next step the bond between C2 and C3 is hydrolyzed in a sterospecific reaction leading to the formation of the L-isomer by the Enoyl-CoA-Hydratase. The L-β-hydroxyl-CoA is oxidized by NAD^+ through the action of the L-β-hydroxyl-CoA-Dehydrogenase which converts the hydroxyl group into a keto group. The final step is the cleavage of the β-ketoacyl-CoA by the β-ketothiolase resulting in an Acetyl-CoA molecule and an acyl CoA molecule that is two carbons shorter. The process is repeated until the entire chain is cleaved into acetyl CoA units. 14 ATP molecules are generated during every oxidation cycle.

The generated Acetyl-CoA can enter the citric acid cycle where Acetyl-CoA is degraded to CO_2. The citric acid cycle produces NADH and $FADH_2$ which are subsequently used in the electron transport chain to produce ATP.

Fatty acid oxidation also occurs in peroxisomes, when the fatty acid chains are too long to be handled by the mitochondria. It is believed that very long chain fatty acids undergo initial oxidation in peroxisomes until octanyl CoA which is followed by mitochondrial oxidation.

In contrast to the mitochondrial β-Oxidation, the FA oxidation in peroxisomes is not coupled to ATP synthesis. Instead, the electrons are transferred to O_2, which yields H_2O_2. The enzyme catalase, found exclusively in peroxisomes, converts the hydrogen peroxide into water and oxygen. Peroxisomal β-oxidation also requires enzymes specific to the peroxisome and to very long fatty acids.

1.4.1 The Carnitine Palmitoyltransferase system

The Carnitine Palmitoyltransferase system allows the transfer of LCFAs into the mitochondria and is composed of three different proteins: CPT1, CPT2 and CACT each with different sub-mitochondrial localization (7, 103). Three CPT1 genes displaying tissue specific expression pattern have been identified in mammals. CPT2 and CACT are ubiquitously expressed throughout the tissues (7, 103).

Deficiencies of the Carnitine Palmitoyltransferase system have been shown to be among the most common causes of inherited fatty acid oxidation disorders.

CACT deficiencies present mostly in the neonatal periods with irregular heart beat, seizures and apnea, and are believed to be triggered by fasting or physiological birth stress. In these patients, free carnitine levels are extremely reduced while long-chain acylcarnitine levels are markedly increased (21, 59, 103).

There are several CPT2 deficiencies described in the literature which can be classified according to the age of onset and tissue distribution of the symptoms. More than 40 mutations of the CPT2 gene locus have been reported to date (21, 59) which are distributed throughout the entire coding sequence. Most of the described mutations are missense mutation (77%) that interfere with the enzymatic activity thus with the level of LCFAs oxidation (21, 59). Indeed, studies have shown that the residual activity of CPT2 activity strongly correlates with the severity of the disease (11, 21, 59). Symptoms are usually triggered by physical exercise, infection and period of fasting and include muscle stiffness and weakness, myoglobinuria, liver failure and transient hepatomegaly (11, 21, 59, 103).

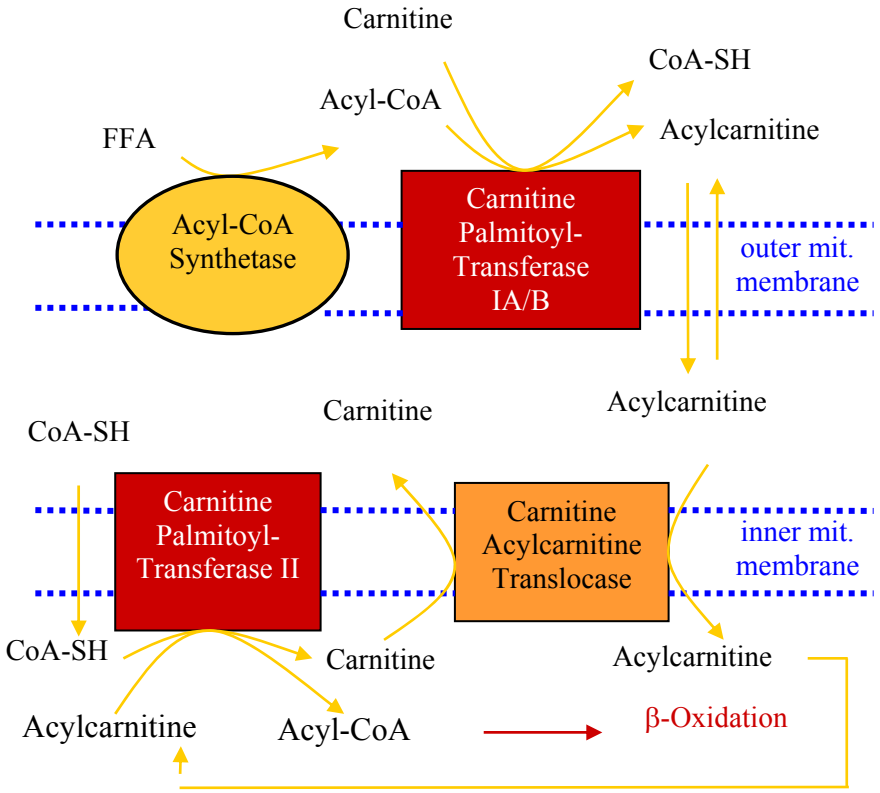


Figure 7: The Carnitine Palmitoyltransferase system.
Upon import into the cell, the FFA is transported to the Acyl-CoA Synthetase where the FFAs is coupled to CoA. CPT1 transfers the fatty acyl group from acyl-CoA to carnitine to allow transport into mitochondria. The CACT exchanges one molecule long-chain acylcarnitine for one molecule of carnitine. CPT2 reverses the reaction of CPT1 to generate the substrate for β-Oxidation.

In most of the Carnitine Palmitoyltransferase system deficiencies the therapy consist in the feeding of a diet rich in carbohydrates and low in fat and the prevention of periods of fasting (21, 103).
CPT1A deficiencies are the only CPT1 deficiency found in humans. CPT1A deficiencies due to mutations of the gene locus are mainly diagnosed in young children after infections or periods of fasting. Symptoms of CPT1A deficiencies include an altered mental status, elevated levels of plasma carnitine and hepatomegaly with or without acute liver failure (59). Up to know no cases of CPT1B or CPT1C deficiencies have been described in the literature. CPT1B expression is crucial for heart function (59). Mutations of this isoform might therefore be incompatible with life (59). Deficiencies of the brain isoform CPT1C if compatible with survival might present as dysregulations in appetite control (59).
Missregulations of CPT1 activity as a regulator of β-Oxidation have additionally been implicated in the development of the metabolic syndrome (21, 43, 73, 143, 209). The metabolic syndrome is mainly characterized by obesity and the insensitivity to insulin signaling and represents a major health problem in our society. In addition to its impact on public health by the means of diabetes, wrong eating habits and obesity are also important factors in the development of some cancers.
Apart from its role in regulating β-Oxidation, CPT1A has also been implicated in cancer development through its link to apoptosis. Nuclear CPT1A expression has been demonstrated

in human tumors where it has been shown to interact with HDAC1. In this context, CPT1A might serve as a regulatory protein that recruits HDAC complexes to acetylated histones of specific genes to silence gene expression by histone hypoacetylation. This contributes to the silencing of tumor suppressor genes and facilitates carcinogenesis (133).

1.4.2 The CPT1 family of proteins

The CPT1 family of protein consists of three proteins that demonstrate a generalized tissue specificity. The three isoforms are encoded by genes located on different chromosomes. Although neither isoform has been isolated in a catalytically active form yet, the physiological roles of CPT1A, the prominent CPT1 gene in liver, and CPT1B, the prominent CPT1 gene in muscle, are well established primarily due to their role in pathogenicity (39, 48, 59).The physiological role of CPT1C, the CPT1 primarily expressed in brain tissues, however is not fully established yet.

CPT1A is the most ubiquitous expresses CPT1 family member and is expressed in liver, pancreas, kidney, blood and brain. CPT1B is only expressed in heart, muscle and brown adipose tissue. CPT1C expression is limited to brain and testis.

The topology of CPT1 within the mitochondrial outer membrane has not been rigorously examined because the tight association of CPT1 with the membrane inhibits the extraction of catalytically active CPT1. Nevertheless, studies have suggested that the catalytic domain and the regulatory malonyl-binding domain are both localized to the cytoplasmic site of the membrane (17, 53, 207).

The control of β-oxidation is manifested at the step of FA import into the mitochondria by regulating the transferase activity of CPT1 in response to the feeding state. CPT1 activity is mainly inhibited by the binding of the allosteric CPT1 inhibitor Malonyl-CoA to the regulatory subunit. During the fed state high insulin levels elevate the Malonyl-CoA concentration sufficiently to inhibit CPT1 activity resulting in a significant reduction in β-Oxidation. In the fasted state in contrast, the Malonyl-CoA level falls and CPT1 inhibition is reversed and β-Oxidation is activated (2, 4, 6, 10, 43, 53, 73, 118, 143). Intracellular Malonyl-CoA concentration is controlled by the activity of two enzymes. It is synthesized by the Acetyl-CoA-Carboxylase (ACC), the rate-limiting enzyme in malonyl CoA synthesis and degraded by the Malonyl-Decarboxylase (MCD). Decreases in the ATP/AMP ratio activate AMPK which phosphorylates ACC and inhibits both its basal activity and activation by citrate, the precursor of its substrate, linking cytosolic Malonyl-CoA synthesis directly to changes in the cellular energy state. Additionally, AMPK activation also leads to the phosphorylation and activation of MCD and subsequent Malonyl-CoA degradation. Due to this regulatory feedback mechanism Malonyl-CoA levels are highest in the fed state and rapidly fall during fasting. As a consequence of decreasing Malonyl-CoA levels CPT1 is reactivated and β-Oxidation rates are increased.

Despite the fact that the CPT1 isoforms are structurally similar, they display significantly different kinetic and regulatory properties. All CPT1 isoforms are inhibited by Malonyl-CoA binding. If compared to CPT1B, CPT1A displays a higher affinity for its substrate carnitine and a lower affinity for Malonyl-CoA (2, 6). The Malonyl-CoA binding efficiency of CPT1C is similar to the binding efficiency of CPT1A. The differences in Malonyl-CoA affinity are believed to be crucial for the function of different cell types by the modulation of tissue partitioning of fatty acid metabolism.

1.5 The Carnitine Palmitoyltransferase 1C

Carnitine Palmitoyltransferase 1 (CPT1) C is an isoform of the CPT1 family that is expressed specifically in the brain where it is enriched in neurons of the hypothalamic nuclei (48). The fact that CPT1C is mainly expressed in the CNS (48, 64, 116, 176, 189), a tissue normally not using fatty acids as a major energy source, suggests a potentially unique function for CPT1C.

1.5.1 CPT1C function in normal brain

CPT1C expression in normal tissue is mainly localized to brain but has also been found in testis (48, 147). In brain, CPT1C expression is found in neurons throughout the entire brain but shows the highest level in neural feeding centers within the hypothalamus (147). Neurons in the hypothalamic feeding centers have been shown to be crucial in appetite regulation and the maintenance of whole body energy homeostasis. Hypothalamic CPT1C expression correlates with the expression of the Fatty Acid Synthetase (FAS), ACC and MCD, enzymes that regulate Malonyl-CoA levels (147). Hypothalamic Malonyl-CoA level serves as an indicator of global energy status and participates in the regulation of feeding behavior. CPT1C expression is also enriched in other brain regions implicated in feeding behavior such as amygdala, a region involved in emotion, reward and food intake (48, 147) and hippocampus, a region important for learning and memory where CPT1C is co-expressed with CPT1A (48, 64).
Cpt1c knock out mice are viable and fertile and display no obvious histological abnormalities through life. This suggests that CPT1C is not required for life or neuronal survival (116, 189).
Cpt1c knock-out in mice of different genetic backgrounds reveal a complex metabolic phenotype. Cpt1c knock-out mice ingest less food and have a lower body weight than wild type (wt) littermates (116, 176, 189). When fed a high fat diet (HFD) the Cpt1c knock-out mice have a markedly increased susceptibility to weight gain although the food intake remains lower than in wt mice (116, 176, 189). Consistent with these results, extopic expression of CPT1C in hypothalamic feeding centers leads to a significant reduction in weight gain in response to a HFD (116, 176, 189) without affecting the food intake. In addition these mice also display a severe insulin resistance in response to HFD (116, 176, 189). Fasting conditions reduce whole body β-Oxidation of Cpt1c knock-out mice if compared to wt littermates while whole body β-Oxidation is equal during ad libitum feeding (116, 176, 189).
The expression of CPT1C in the hypothalamic feeding centers as well as the metabolic phenotype observed in the knock-out mouse suggest for a regulatory rather than a metabolic role of CPT1C and an involvement of CPT1C in peripheral energy sensing and homeostasis. The molecular pathways underlying this phenotype nevertheless need to be further investigated. In contrast to the Cpt1c knock-out mouse where no morphological changes have been observed, murine embryonic stem cells (ES cells) depleted of CPT1C ($Cpt1c^{gt/gt}$) display significant changes in mitochondrial morphology if compared to $Cpt1c^{wt/gt}$ and $Cpt1c^{wt/wt}$ ES cell control. These cells contain swollen mitochondria that display an abnormal internal membrane structure and a loss of cristae structure (236). Additionally, these cells exhibit a reduction in cell diameter and cell survival due to caspase 3 and 9 activation. $Cpt1c^{gt/gt}$ ES cells show an abnormal accumulation of lipid droplets in the cytoplasm and furthermore display significant changes in the intracellular lipid profile (236). Loss of Cpt1c function in ES cells has been shown to decreased cell survival in response to hypoxia and glucose deprivation (236).
In contrast to the metabolic phenotype observed in Cpt1c knock-out mice, the intracellular localization as well as the catalytic activity are controversial in the literature (116, 170, 176).

It has been shown that CPT1C like CPT1A and CPT1B is expressed in the mitochondria of cultured neurons (48, 116).
In contrast to this result, a different publication showed that CPT1C expressed in the endoplasmatic reticulum in fibroblasts transiently transfected with CPT1C (170). Additionally, while different studies failed to show a catalytic activity of CPT1C (116, 176), one study showed that CPT1C has carnitine acylcarnitine transferase activity upon constitutive expression in neuronal PC-12 cells (170). Constitutive expression of CPT1C in PC-12 cells however, fails to increase β-Oxidation rates (170). Further studies are necessary to solve this controversy and to further elucidate the molecular function of CPT1C in neurons.

1.5.2 CPT1C function in cancer

Very little is known about the role of CPT1 family members in carcinogenesis. Nevertheless, the implication of CPT1C in the hypothalamic regulation of peripheral energy expenditure (116, 117, 147) and ES cell survival in response to hypoxia and glucose deprivation (236) make it a worthwhile target for cancer-related studies.
It is well established that obesity is a major risk factor for the development of various cancers (WHO, 102, 108, 208). Although the cellular and molecular pathways involved in this process are poorly understood, the link between obesity and carcinogenesis has been found by several studies during the last years (102, 108, 177, 209).
Activation of metabolic pathways by metabolic stress like hypoxia or missregulation by mutations have been shown to contribute to tumor development (WHO), metabolic adaptation of tumor cells (86, 102, 218, 220) and metastasis (220, 228). The metabolic adaptation of tumor cells is mainly believed to function through changes in glucose metabolism. Recent studies nevertheless show that FAs serve as an important energy source in some cancer types.
Up to now only one study has been submitted that implicates changes in CPT1C expression to cancer development (236). The analysis of CPT1C mRNA expression in lung cancer samples shows that CPT1C is overexpressed in most of the lung tumor samples. The same study showed that CPT1C expression in established cancer cell lines is controlled by pathways that have been shown to contribute to carcinogenesis. CPT1C is a direct transcriptional target of p53, which is recruited to the CPT1C gene locus in response to glucose deprivation and hypoxia. Furthermore, CPT1C mRNA expression is induced by hypoxia and AMPK activation upon glucose starvation.
Consistent with the results obtained with Cpt1c-deficient ES cells (236), CPT1C-depleated established cancer cell lines display a reduction of cell growth under hypoxic conditions as well as reduced tumor growth in Xenograft assays. Tumor growth was further reduced by treatment with the AMPK inhibitor Metformin. While cells depleted from CPT1C display a reduction in ATP production, constitutive expression of CPT1C results in significantly increased levels of ATP production and FAO. Additional studies are necessary to validate these experiments and to elucidate the role of CPT1C in carcinogenesis and the mechanisms underlying the phenotypical changes induced by CPT1C depletion or over-expression. Elucidating the mechanism by which CPT1C influences the sensitivity to hypoxia may provide a novel approach to improve the prognosis of hypoxic and therefore treatment-resistant cancers.

2 The CPT1C 5'UTR contains a repressing upstream open reading frame that is regulated by cellular energy availability and AMPK

Ines Lohse[1], Patrick Reilly[1,2], Kathrin Zaugg[1]

Department of Radiation Oncology, University Hospital Zurich, Switzerland
Department of Cellular and Molecular Research, National Cancer Centre Singapore, Singapore

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I. Lohse*, P. Reilly#, K.Zaugg*
* Department of Radiation Oncology, University Hospital Zurich, Zurich, Switzerland
# Department of Cellular and Molecular Research, National Cancer Centre Singapore, Singapore

Abstract
Translational control is utilized as a means of regulating gene expression in many species. In most cases, posttranscriptional regulatory mechanisms play an important role in stress response pathways and can lead to dysfunctional physiology if blocked by mutations.
CPT1C, the brain-specific member of the CPT1 family, has previously been shown to be involved in regulating metabolism in situations of energy surplus. Sequence analysis of the CPT1C mRNA revealed that it contains an upstream open reading frame (uORF) in the 5' UTR of its mRNA. Using CPT1C 5' UTR/luciferase constructs, we investigated the role of the uORF in translational regulation.
The results presented here show that translation from the CPT1C main open reading frame (mORF) is repressed by the presence of the uORF, that this repression is relieved in response to specific stress stimuli, namely glucose deprivation and palmitate-BSA treatment, and that AMPK inhibition can relieve this uORF-dependent repression. The fact that the mORF regulation is relieved in response to a specific set of stress stimuli rather than general stress response, hints at an involvement of CPT1C in cellular energy-sensing pathways and provides further evidence for a role of CPT1C in hypothalamic regulation of energy homeostasis.

Introduction
The presence of upstream open reading frames (uORF) within mRNA 5´UTR can impact levels of translation initiation of the main open reading frame (mORF). Because eukaryotic ribosomes generally only initiate once per mRNA (1), the presence of an uORF normally inhibits the translation of the mORF and may lead to mRNA decay (2, 3, 4). Although they are usually short sequences, uORFs may still play critical roles in modulating physiology. Indeed, mutations that introduce new or disrupt existing uORFs have been reported to cause human diseases (5, 6, 7, 8).
Carnitine Palmitoyltransferase 1 (CPT1) C is an isoform of the CPT1 family that is expressed specifically in the brain under normal conditions (9). It is well established that CPT1A and CPT1B, the other two CPT1 family members, catalyze the initiating step of fatty acid degradation through which long chain fatty acidy are transported from the cytoplasm to the mitochondrial matrix for β-oxidation (10, 11). In this enzymatic reaction, the fatty acyl group is transferred from acyl-CoA to carnitine to allow transport into mitochondria. The fact that CPT1C is mainly expressed in the CNS (9, 12), a tissue normally not using fatty acids as a major energy source, suggests a potentially unique function for CPT1C. Recent publications show that CPT1C expression in the brain is mainly restricted to the hypothalamic feeding centres, where lipid metabolism is believed to play a key role in regulating peripheral energy homeostasis. Results derived from studies using Cpt1c knock out mice implicate CPT1C in the regulation of energy homeostasis and the control of food intake (13, 14). Although Cpt1c has been shown to be involved in hypothalamic energy sensing, its molecular function, regulation and signalling pathways still remain controversial (12, 14, 15, 16).
Analysis of the mRNA sequences revealed that CPT1C is the only CPT1 member that contains an uORF and that the presence of CPT1C uORFs is conserved in several mammalian species. Experiments using luciferase reporter constructs transfected into brain-derived cell lines reveal that the presence of the uORF inhibits the translation of the downstream mORF.

In addition, these experiments showed that the translation inhibition by the uORF is derepressed in situations of glucose deprivation and palmitate-BSA treatment.

Material and methods

Computational analysis of the CPT1C mRNA

RNA secondary structure was calculated with the help of the MFOLD program version 3.2 from the Michael Zucker homepage (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi). Calculations were performed with default settings.

Plasmid construction

The 5`UTR of the CPT1C transcript was amplified from the cDNA clone (MGC, Invitrogen) by PCR under standard conditions using the following primer (restriction sites are underlined) fwd 5`-CTC GAG GGA ATC GGG GTT TCT GGG TGA CGG-3`and rev 5`-AAG CTT GTC ACG CTG GAG CCC ACG and cloned into a TA vector (Invitrogen). To create a construct lacking a functional start codon in addition to the wt 5´UTR construct, the ATG of the uORF was mutated to ACG with the following primers fwd 5`-GGC ATT GGA CAT ACG CAA GCG GGA G-3´ and rev 5´-CTC CCG CTT GCG TAT GTC CAA TGC C-3´ using a standard side directed mutagenesis PCR. After sequencing, the XhoI/HindIII restricted inserts of both constructs were subcloned into the phCMV-Cluc-FSR vector (Genlantis).

Preparation of Fatty Acid-BSA complexes

20% essentially fatty acid free BSA was prepared in 150mM NaCl and sterile filtered. 20mM fatty acid was sapoficated and dissolved in 150mM NaCl heated up to 65°C. The free fatty acid was complexed to BSA by adding an equal volume 20% BSA to the 65°C fatty acid solution and stirred for 10min. The solution was allowed to cool down under stirring and sterile filtered before alliquoting.

Cell culture

The human Glioblastoma cell lines T98G and U87-MG and the SV40 large T-Ag immortalized fetal human non-neoplastic astrocytic cell line SV40-FHAS were purchased from the American Type Culture Collection (ATCC).

The cells were maintained in monolayer culture in DMEM containing 4.5g of glucose supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin. All cells were grown in a humidified atmosphere of 5% CO_2 at 37°C.

For hypoxic conditions, the T98G cells were cultured at 37°C with 5% CO_2, 94% N_2 and 0.2% O_2 in a hypoxic incubator (Scholzen AG). For all fatty acid treatments the supplements were added to the normal growth medium. For the starving conditions either DMEM or DMEM without glucose supplemented with 1% L-Glutamine and 1% Penecillin/Streptomycin with and without 10% FBS was used.

Luciferase assay

T98G cells were co-transfected with the firefly luciferase constructs and the pRL-SV40 renilla luciferase vector (Promega) using Lipofectamine 2000 (Invitrogene) and seeded 12h prior to treatment with 10^4 cells in 96 Micro-Assay-Plates (Greiner-bio-one). Relative luciferase levels were determined by the ratio of firefly luciferase and renilla luciferase activity. The luciferase activities were measured by the Dual-Glo Luciferase System (Promega) according to the Dual-Glo Luciferase System manual (Promega) using the Glomax 96-well luminometer (Promega). All experiments were repeated at least 3 times in triplicates and included Mock transfections and transfections with the phCMV-cLuc-FSR vector.

Results

Human CPT1C mRNA contains an uORF upstream of the mORF.

The CPT1C gene consists of 21 exons that give rise to two transcript variants. Although both mRNAs are transcribed from the same gene, both transcript variants differ in lengths and exon content. In both transcript variants exon 2 and parts of exon 16, and in transcript variant 2 also exon 3, are not part of the spliced mRNA. These exons, although not part of the mRNA might, however, have a regulatory function for either transcriptional or posttranscriptional regulation mechanisms.

Both mRNAs, however, contain one uORF maintaining the same start codon but stop codons derived from exon 3 and exon 4 respectively. The uORF within transcript variant 1 starts at bp 97 and has a stop codon at bp 198 resulting in a 34 AA long peptide. The uORF within transcript variant 2 on the other hand starts at bp 91 and the corresponding stop codon at bp 206 resulting in a 37 AA long peptide. Neither of the peptides produced by the uORFs has any similarity to known proteins or protein domains. A computational RNA folding prediction using MFOLD revealed that although both transcript variants show significantly different folding patterns at 37°C, the uORF ATG of both transcript variants are located in bubble-like structures while the ATG of the mORF are located in double-strand sections (data not shown).

CPT1C is the only CPT1 family member containing an uORF upstream of the mORF.

CPT1C mRNA sequence analysis revealed that the presence of an uORF is conserved in CPT1C mRNAs of different species but not within the CPT1 family of proteins. CPT1C mRNA sequences of various species listed on NCBI showed that all mRNAs contain long 5´ UTRs harbouring one or several uORFs (Table 1). The sequences and number of the mammalian CPT1C uORFs can vary significantly suggesting that the subsequent peptide(s) are not functional. For example, whereas the human CPT1C mRNA contains a single uORF out of frame with the mORF, the rat CPT1C mRNA contains three uORF´s that are located both in frame as well as out of frame with the mORF. It should be noted that no paralogs of CPT1C have been annotated as such for non-mammalian vertebrates. To determine whether the presence of an uORF is also a common feature of the whole CPT1 family, we analyzed the mRNA sequences of the other two CPT1 family members, CPT1A and CPT1B. This analysis, however, revealed that both mRNAs have short 5´ UTRs containing no similar uORFs (Table 1). Indeed no annotated mammalian CPT1A or CPT1B paralog demonstrated an uORF (data not shown). This conservation suggests the CPT1C uORFs may be functional components of the mRNA that can regulate translation of the mORF. MFOLD analysis of CPT1C paralogs indicate that although they do not share common mRNA folding pattern, the part that contains the uORF ATG is located in a bubble-like structure similar to human CPT1C (Figure 1A).

Table 1: ***uORF distribution and 5´UTR length throughout the CPT1 family***

species		5´UTR length	no. uORF´s
human	CPT1A	170bp	0
human	CPT1B	84bp	0
human	CPT1C transcript variant 1	281bp	1
human	CPT1C transcript variant 2	206bp	1
mus musculus	cpt1c	194bp	2
rattus norwegicus	cpt1c	199bp	3
bos taurus	cpt1c	225bp	2
pogo abelii	predicted cpt1c	228bp	1

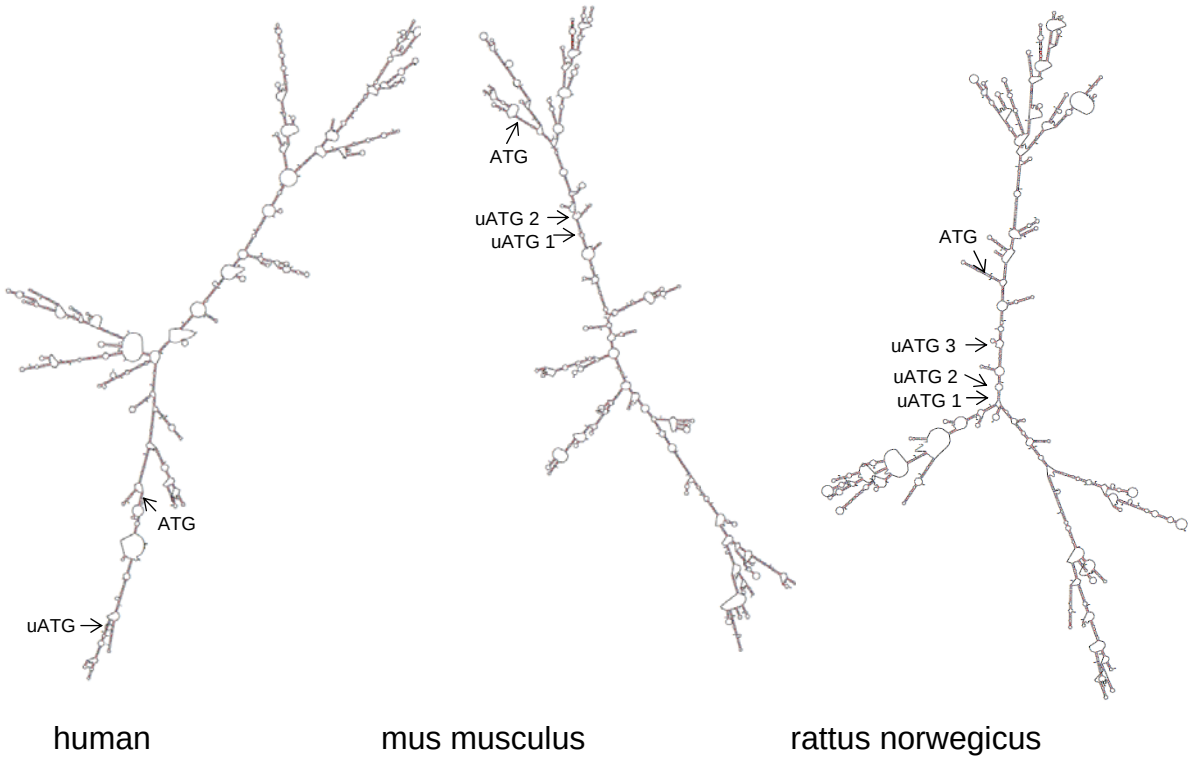


Figure 1: ***CPT1C uORFs are conserved in evolution and share structural elements.*** Secondary mRNA structure of CPT1C paralogs. Folding was performed using MFOLD with default parameters at 37°C. uATG, uORF initiator codon, ATG, mORF initiator codon.

Effects of the CPT1C uORF on the translation of the downstream mORF

Short uORFs can function as regulatory elements on the level of translation. The presence of the uORF in both CPT1C transcript variants suggests that both variants can be regulated by the same mechanism (Fig. 1A). Because of this similarity between the transcript variants, we chose to only use the 5`UTR of CPT1C transcript variant 1 for further experiments. To analyse the uORF function, we cloned the CPT1C 5'UTR upstream of a luciferase reporter from a heterologous CMV immediate early promoter. As a negative control for non-uORF activities of the 5'UTR, we also cloned the same sequence where the start codon ATG of the uORF was mutated to ACG (Fig. 2A). Transfection of glioblastoma-derived T98G cells demonstrated that the 5´UTR containing the mutated start codon exhibit a 3-fold higher luciferase activity than cells transfected with the 5´UTR containing the start codon (Fig. 2B). SV40-FHAS and U87-MG cells transfected with the plasmids contained the mutated uORF

also had an increase in luciferase activity (2.5- and 2.6-fold, respectively) when compared to the wild type (wt) luciferase reporter construct (data not shown). The difference in translation efficiency was observed 24h as well as 36h after transfection. These data indicate that the presence of the intact uORF of the CPT1C mRNA inhibits the translation of the mORF under normal growth conditions.

CPT1C mORF translation is increased in response to glucose deprivation

Most of the previously examined uORFs have been shown to regulate the translation of the mORF in response to the cellular environment. In order to test whether different metabolic stress stimuli influenced CPTIC 5'UTR activity, we treated the T98G, SV40-FHAS and U87-MG cells transfected with the wt uORF luciferase reporter constructs in altered cellular environments.

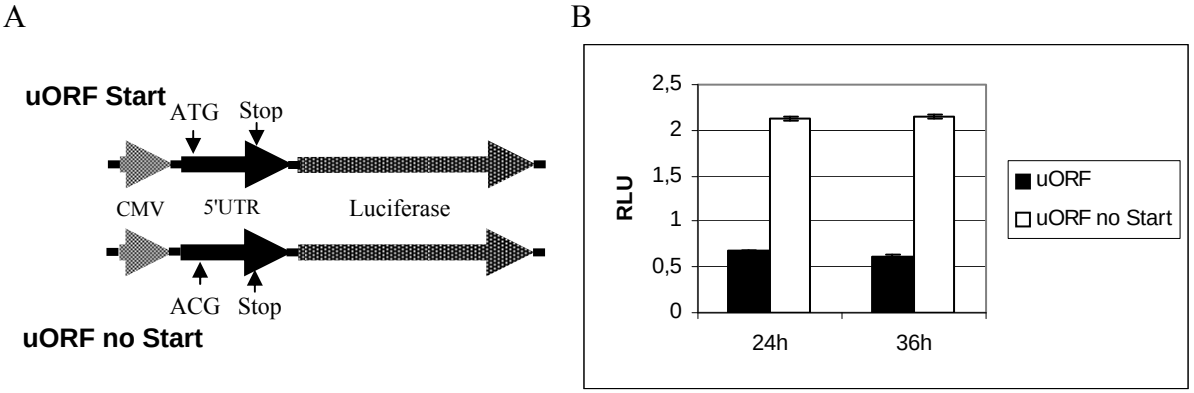
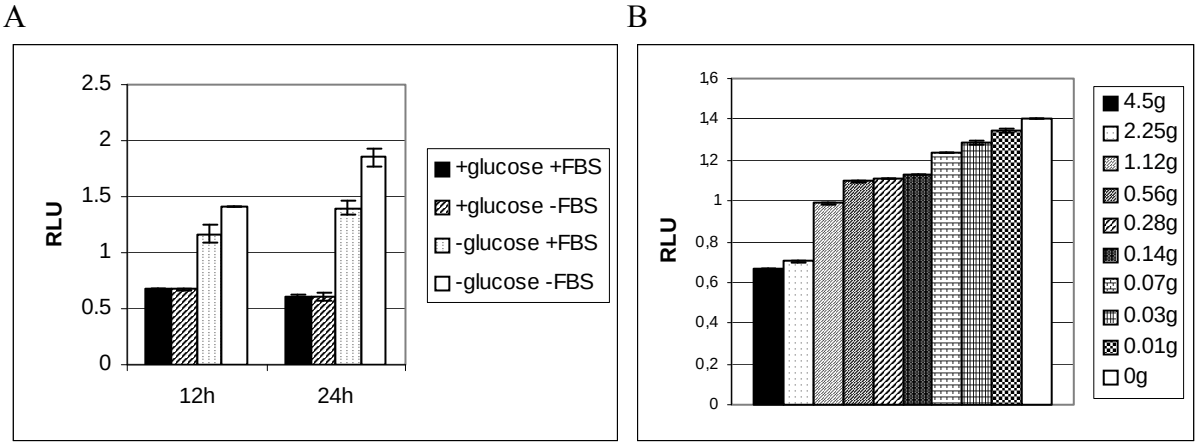


Figure 2: ***CPT1C 5'UTR induces reporter gene repression.***
(A) Reporter gene constructs containing the CPT1C 5´UTR cloned in front of a luciferase reporter gene with either the endogenous uORF start codon (ATG) or the mutated uORF start codon (ACG). (B) Luciferase activities of uORF start (filled bars) and uORF no start (empty bars) reporters in T98G cells after transfection and maintenance in normal conditions for 24h or 36h. *RLU,* Relative Luciferase Units.

To examine the influence of glucose limitation on the uORF-mediated translational repression, the cells were incubated in media lacking glucose and/or FBS. In T98G cells the luciferase activity was increased by 1.5 fold after 12h and by 2-fold after 24h glucose deprivation (Fig. 3A) compared to cells grown in normal growth medium. Alone, FBS deprivation had no effect on luciferase activity at either time point (Fig. 3A). In the absence of glucose, however, FBS deprivation did lead to an increase of 2-fold after 12 and 3-fold after 24h treatment in T98G cells (Fig. 3A) when compared to cells grown in normal growth medium. The change of luciferase activity in SV40-FHAS and U87-MG cells upon treatment with the described deprivation conditions were similar to the changes observed in T98G cells (data not shown). To exclude that increased luciferase activity in response to glucose deprivation was derived from treatment-induced changes in transcriptional activity, cells transfected with the mutated 5'UTR construct were treated in parallel to the cells transfected with the wt 5'UTR constructed. In the absence of the intact ATG, we did not see any apparent changes in luciferase activity in response to the deprivation media (Fig. 3C), suggesting that the derepression by is not transcriptionally regulated but, rather, dependent on the uORF.
To elucidate at what glucose concentration the CPT1C 5'UTR loses repression from the regulation by the CPT1C uORF, we performed a glucose titration on the transfected T98G cells. Because we saw little effect from the absence of FBS, we performed the glucose titration in the presence of FBS. The first strong increase in firefly luciferase activity can be

observed at the titration step containing 1.125g of glucose with a stepwise increase of activity until the highest activity is reached in the medium containing no glucose. The general trend of slow increase in luciferase expression, however, suggests that glucose responsiveness of the CPT1C 5'UTR is not tight switch but rather a concentration sensor of glucose or a glucose-dependent metabolite.

To exclude that the CPT1C 5'UTR is derepressed in response to general stress pathway activation rather than mediating a specific stress response, the cells were treated with 0.2% O_2 for 12h with or without a 12h reoxigenation period following the hypoxic conditions. None of the tested hypoxia or reoxigenation conditions led to changes in reporter activity in T98G cells (data not shown).



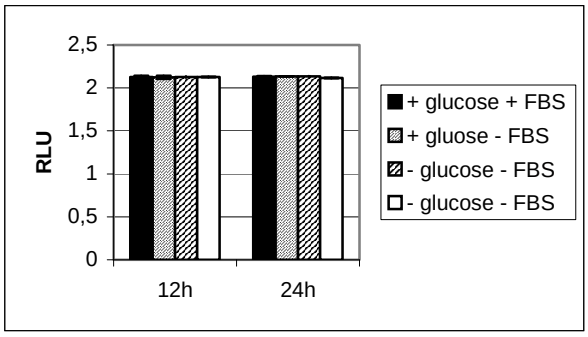


Figure 3: ***Modulation of CPT1C 5'UTR repression by varied growth conditions.*** Relative luciferase activities of T98G cells transfected with the wt uORF reporter gene construct after (A) glucose and/or serum deprivation for 12h or 24h; and with (B) titration of glucose concentration in media after 12 hours. (C) Relative luciferase activities of T98G cells transfected with the no start uORF reporter gene construct after glucose and/or serum deprivation for 12h or 24h.

CPT1C uORF regulation is influenced by AMPK

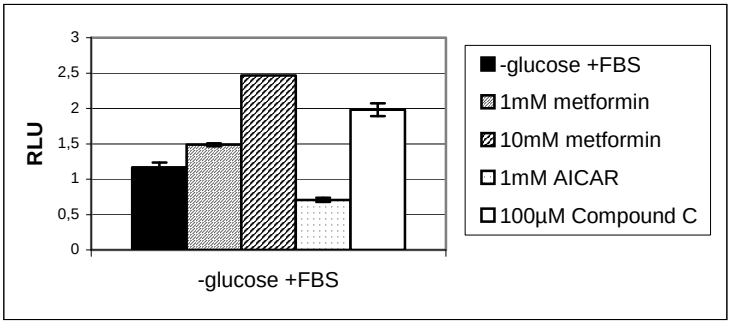
To test whether the derepression by the CPT1C 5'UTR in response to the cellular environment is dependent on the activation of AMPK, the transfected cells were treated with chemical AMPK activators and inhibitors. Furthermore, the mTOR inhibitor Rapamycin was used investigated an involvement of mTOR in potential uORF regulation.

T98G cells were treated with the different compounds in combination with two different deprivation media. The results presented in Figure 3 revealed that the CPT1C uORF is sensitive to glucose but not to FBS deprivation. Glucose deprivation leads to low energy levels and high intracellular AMP and thus to the activation of AMPK, which starts a signalling cascade that aims to protect the cell from metabolic stress (17).

Treatment with metformin, an inhibitor of hypothalamic AMPK, with and without glucose deprivation led to an increase in luciferase activity in a dose-dependent manner. T98G cells

treated with 1mM or 10mM Metformin showed to a 1.2- or 2-fold increase, respectively, in reporter activity under glucose deprivation conditions (Fig. 4A) and a 1.6- or 3.6-fold increase, respectively, if treated with FBS deprivation (Fig. 4B). Consistent with AMPK positively activating the CPT1C 5'UTR repression mechanism, treatment with the AMPK agonist AICAR decreased reporter activity under glucose deprivation conditions but not in FBS deprivation conditions (Fig. 4A, B).

A



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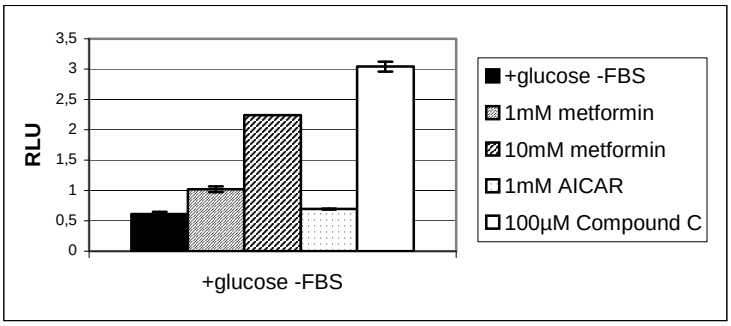

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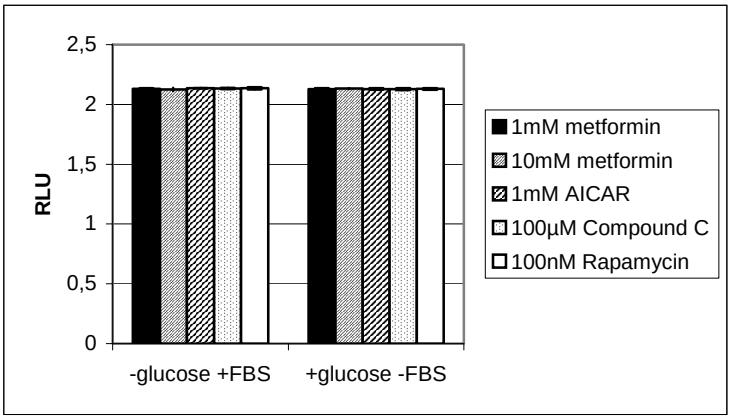
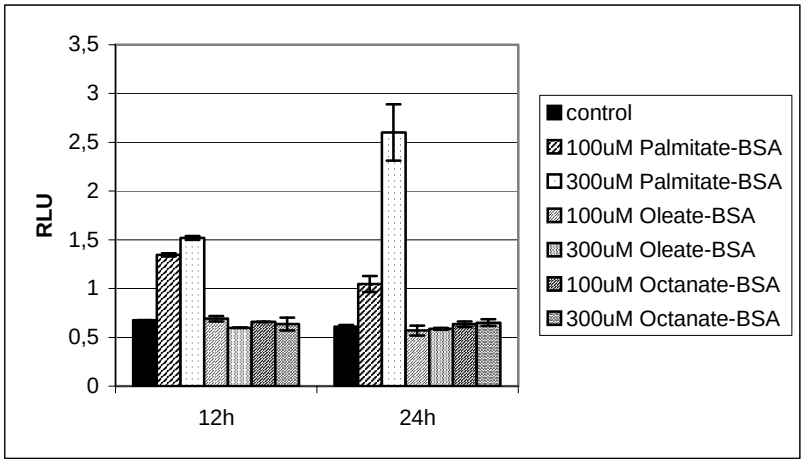


Figure 4: ***CPT1C 5'UTR repression is relieved by AMPK activation.*** Relative luciferase activity in T98G cells transfected with uORF start reporter construct after maintenance in media containing either (A) FBS but no glucose or (B) glucose but no FBS in the presence of AMPK-inhibitors metformin or Compound C, or in the presence of the AMPK-activator AICAR. (C) Relative luciferase activity in T98G cells and transfected with uORF no start reporter construct after maintenance in the presence of AMPK-activators metformin or Compound C, the AMPK-inhibitor AICAR, or mTOR-inhibitor Rapamycin

The AMPK-specific inhibitor, Compound C, also induced a derepression of the 5'UTR consistent with that seen for metformin. Curiously, with this compound, higher derepression of the reporter activity was seen in the FBS deprivation than in glucose deprivation conditions. The increase in luciferase activity was a 4.9-fold increase in FBS deprivation (Fig. 4B), if compared to a 1.7-fold increase in cells treated with glucose deprivation (Fig. 4A). We suggest that this effect is likely due to different pharmacodynamics of Compound C in the presence of serum. Again, similar results were seen with the alternate cell line SV40-FHAS (data not shown). Analogous to the results shown in Figure 3C, treatment with the pharmacological inhibitors in combination with the deprivation media did not affect the luciferase activity in cells transfected with the mutated 5'UTR construct containing no Start

codon (Fig.4C). This suggests that AMPK activity is likely to be very important in regulating this translational derepression of CPT1C.
Inhibition of mTOR by the specific inhibitor Rapamycin did not influence the CPT1C uORF in neither of the tested cell lines under either of the deprivation conditions (data not shown). This suggest that the CPT1C 5'UTR is responsive to energy supply and not responsive stimuli that mimics amino acid deprivation.

A



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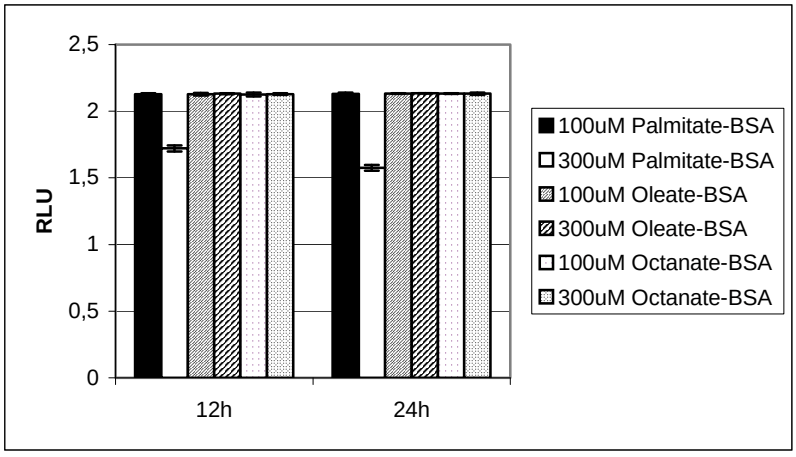

Figure 5: ***Palmitate relieves CPT1C 5'UTR-mediated translational repression.***
Relative luciferase activity of (A) uORF start and (B) uORF no start reporter constructs after transfection into T98G cells upon treatment with stated BSA conjugated fatty acids for 12h or 24h. RLU, Relative luciferase units, Palmitate-BSA, Palmitic acid conjugated BSA, Oleate-BSA, Oleic acid conjugated BSA, Octanate-BSA, Octanaic acid-conjugated BSA.

Palmitate-BSA treatment releases the CPT1C mORF from the uORF
It has been established that CPT1C like the other CPT1 family members binds long-chain fatty acids (LCFA), although the enzymatic activity of the CPT1C transferase domain is still controversial in the literature (12, 14, 15, 16). Intracellular accumulation of saturated LCFA, for example palmitate, in non-adipose tissue leads to an inhibition of proliferation and apoptosis (de Pablo, 1999; Mayer, 2010). In cell culture, treatments with different LCFAs induce cells stress, whose consequences vary between cell lines and FA concentrations. Palmitic and oleic acid are both LCFA and known substrates of CPT1A and CPT1B, whereas CPT1C proteins do not catalyze import of short chain fatty acid, for example oleic acid, into

the mitochondria (11, 18). To evaluate the impact of palmitic acid, oleic acid and octanoic acid on the regulation of the mORF by the uORF, the transfectants were treated with either 100µM or 300µM FA-BSA. In normal glucose conditions, treatment with 100µM Palmitate-BSA for 12h led to a 1.9-fold increase in luciferase activity in T98G cells (Fig. 5.A) and 2.6-fold in SV40-FHAS cells (data not shown), if compared to the untreated control.
Here, the increase in luciferase activity was not maintained over a treatment period of 24h possibly due to substrate decomposition. After 12h of treatment, 300µM Palmitate-BSA led to a small further increase over 100µM treatment levels. At this concentration, however, a further increase (5-fold above normal) could be seen after 24h of treatment. This is equivalent to a 4-fold increase over 100µM Palmitate-BSA treatment (Fig. 5A). Furthermore, SV40-FHAS cells exhibited an 8-fold increase over normal conditions after 24h of treatment (data not shown). Treatment with neither oleic or octanioc acid at the tested FA concentrations resulted in increased luciferase activity (Fig. 5A). In contrast to the results shown in Figure 3 and 4 where none of the treatments did affect the luciferase activity in cells transfected with the mutated 5'UTR, treatment with 300µM Palmitate-BSA did lead to a significant decrease of luciferase activity, which suggest a decrease of transcription in response to the treatment. This decrease was not observed when the cells where treated with 100µM Palmitate-BSA or one of the other two FA at either concentration (Figure 5B). The reduction of transcriptional activity seen in the 300µM Palmitate-BSA treated cells suggests that the translational derepression is stronger than indicated by the fold change in the wt 5'UTR transfected cells (Figure 5A). These data suggest that there is a concentration sensing and a time-dependent sensing mechanism used for derepression of the CPT1C 5'UTR.

Discussion

The above data suggest that CPT1C can be regulated through an uORF and that this regulation can be impacted by cellular energy availability and AMPK activity.
Although CPT1C has structural similarities to the other CPT1 isoforms and binds malonyl-CoA, neither its enzymatic activity nor its substrate(s) are established (12, 14, 15, 16).
Sangiao-Alvarellos et. al recently showed that a reduction of CPT1C mRNA expression does not necessarily lead to a reduction of CPT1C protein level. While CPT1C mRNA expression was markedly decreased in hypothalamic preparations of Dwarf rats, a growth hormone deficient rat strain, the CPT1C protein level was not significantly changed compared to the wild type control (19).
The finding that transcriptional as well as translational mechanisms are used to control CPT1C suggests that CPT1C expression needs to be tightly regulated in hypothalamus. This observation adds further evidence to our hypothesis that the uORF-dependent regulation of CPT1C translation plays an important role in the regulation of CPT1C protein level *in vivo*.
Our results indicate that uORF regulation within the 5′UTR maintains low basal CPT1C expression during unstressed conditions and is important in the translational induction for expression during reduced energy availability.
Regardless of the unknown function of CPT1C in hypothalamus, the post-transcriptional regulation suggests that CPT1C gene expression must rapidly change to perform its role. Combined with the fact that the Cpt1c gene-deficient mouse demonstrates a metabolic phenotype (12), there is increasing evidence that CPT1C gene expression may be an important AMPK effector in regulating satiety.
Independent of the increasing evidence suggesting that this uORF-dependent translation regulation of CPT1C protein expression might play a role in hypothalamic energy sensing, the role of CPT1C within the complex hypothalamic signalling network still needs to be elucidated.

Further biochemical studies will be necessary to elucidate the factors through which AMPK activation may control this translational derepression and whether AMPK and BSA-Palmitate may be functioning through a common mechanism.

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3 CPT1C Is Upregulated by Transductions with an HIV-Based Lentiviral RNAi Delivery System

I. Lohse*, RF. Speck#, K.Zaugg*

* Department of Radiation Oncology, University Hospital Zurich, Zurich, Switzerland
# Division of Infectious Diseases and Hospital Epidemiology, University of Zurich, University Hospital Zurich, Zurich, Switzerland

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CPT1C Is Upregulated by Transductions with an HIV-Based Lentiviral RNAi Delivery System

I. Lohse*, RF. Speck#, K.Zaugg*

* Department of Radiation Oncology, University Hospital Zurich, Zurich, Switzerland
# Division of Infectious Diseases and Hospital Epidemiology, University of Zurich, University Hospital Zurich, Zurich, Switzerland

Abstract
Commercially available lentiviral systems are widely used tools for stable gene delivery into cell lines and tissues. Although they have long been understood to cause insertional artefacts, they are generally believed to be relatively inert on the physiology of the transduced cell, Here, we report that transduction of established human cell lines with lentiviral vectors leads to a dramatic increase in the Carnitine Palmitoyl Transferase (CPT1) C expression. We further demonstrate that this consequence is likely due to HIV components in these vectors. These observations suggest that lentiviral based transduction may induce changes of cell metabolism due to CPT1C mRNA upregulation.

Introduction
Carnitine Palmitoyltransferase 1 (CPT1) C is an isoform of the CPT1 family that is expressed mainly in the brain under normal conditions (1). It is well established that CPT1A and CPT1B, the other two CPT1 family members, catalyze the initiating step of fatty acid degradation through which long chain fatty acids are transported from the cytoplasm to the mitochondrial matrix for β-oxidation (2). In this enzymatic reaction, the fatty acyl group is transferred from acyl-CoA to carnitine to allow transport into mitochondria. The fact that CPT1C is mainly expressed in the central nervous system (CNS) (1, 3, 4), a tissue normally not using fatty acids a as a major energy source, suggests a potentially unique function for CPT1C. Recent publications show that CPT1C expression in the brain is mainly restricted to the hypothalamic feeding centers, where lipid metabolism has been shown to play a key role in regulating peripheral energy homeostasis. Results derived from studies using CPT1C knock-out mice implicate CPT1C in the regulation of energy homeostasis and the control of food intake (3, 4, 5).
The use of lentiviral gene delivery systems enables rapid gain- and loss-of-function analysis in cells. These lentiviral gene delivery systems are usually based on the generation of pseudotype viruses consisting of 1) the envelope protein from the vesiculo-stomatitis virus (VSV) which permits attachment of the pseudotype virus to a broad spectrum of cell types, 2) the matrix protein from the human immunodeficiency virus (HIV) type-1, gag-pol, which includes HIV's own enzymes that mediate reverse transcription and integration of the encapsidated RNA, and 3) two copies of the gene of interest, i.e. the encapsidated RNA, under the control of HIV's long-terminal repeat (LTR) which contains also the necessary packaging signals. The system used for our experiments contains all these genetic information on three different vectors resulting in the production of pseudotype viruses which are replication incompetent. Once infected, the gene of interest is reverse transcribed and subsequently integrated into the host cell genome without any further production of infective particles (6).
Our efforts to generate an *in vitro* loss-of-function system for CPT1C lead us to the surprising finding that CPT1C mRNA was induced by lentivirus. This observation appears to be specific in that neither family members CPT1A nor CPT1B were induced.

Material and Methods

Plasmid construction

The shRNA sequences directed against CPT1C and Lamin A (LMNA) as well as an unspecific negative control shRNA (Tab. 1) were designed using the BLOCK-iT RNAi designer, synthesized and annealed to the miR RNAi sequence. Four different siRNAs were tested for CPT1C and the most efficient sequence was chose for the knock down experiments (sequence information upon request). The generated shRNA was inserted into the pcDNA6.2-GW/EMGFP-miR vector and the sequence was verified by sequencing the corresponding fragment. For transduction of the shRNA sequences, the shRNAmir cassette was transferred into the pLenti6.2-V5DEST vector which can be packed into viruses produced with the ViraPower ™ Lentiviral expression system (Invitrogen). The pcDNA6.2-GW/EMGFP-miR constructs were used for shRNAmir delivery by transfection.

Table 1: *shRNA sequences*

The shRNA sequence forms a hairpin structure that is processed into a mature miRNA (bold) while the loop (underlined) and the miRNA* (italics) are degraded.

gene	shRNA sequence (5`-`3)
Scrambled control	**AAATGTACTGCGCGTGGAGAC**GTTTTGGCCACTGACTGAC*GTCTCCACGCAGTACATTT*
LMNA	**TGGAAGTCCAGTTCCTCCTTC**GTTTTGGCCACTGACTGAC*GAAGGAGGCTGGACTTCCA*
CPT1C	**AGAAGATGCGGACCAGGGCCA**GTTTTGGCCACTGACTGAC*TGGCCCTGCCGCATCTTCT*

Virus production

Replication-deficient HIV-based lentivirus for the shRNAmir delivery was produced using the ViraPower ™ Lentiviral expression system (Invitrogen) according the to manufacturer's instructions.

Cell culture

The human cell lines T98G, U87-MG, MCF7 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC). The cells were maintained in monolayer culture in DMEM supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin. All cells were grown in a humidified atmosphere of 5% CO_2 at 37°C.

For stable cell line generation cells were either infected with the produced replication-deficient virus or transfected with cDNA6.2-GW/EMGFP-miR using Lipofectamine 2000 (Invitrogen) (7) according to the manufacture's manual or infected with an MOI of 1. Stable clones were selected under antibiotic selection with 8µg/ml Blasticidine after 14 days and pools of the generated stable clones were used for analysis.

TZMBL cells, a HELA derived cell line were infected with HIV and served as a positive control for HIV infection-related effects.

Quantitative RTPCR Analysis

Total RNA was extracted from cells with the Trizol Plus Mini-to-Midi RNA extraction kit (Invitrogen) and cDNA was prepared using the Superscript III cells direct cDNA synthesis kit (Invitrogen) according to the manufacturer's manual. qRT PCR was performed using the SYBR Green qPCR Super-Mix UDG kit (Invitrogen). GAPDH was used as an internal reference. Relative RNA levels were calculated by the ΔΔCt method (8). The following primers were used: fwd 5'- ATG ACA TCA AGA AGG TGG TG -3' and rev 5'- CAT ACC AGG AAA TGA GCT TG -3' for GAPDH; fwd 5'- TGG AAC TCA GTG CCC CTG TG -3' and rev: 5'- GCA GGA AAC ACA CCG GTG AG -3' for CPT1C; fwd: 5'- AGA AAT GTC

GCA CGA GCC CAG AC -3' and rev: 5'- CCA TGG CCC GCA CGA AGT C –3' for CPT1A; fwd: 5'- CTT TGG CCC TGT AGC AGA TGA -3' and rev: 5'- TGC TCT CTG AGC TTG AGA ACT T -3' for CPT1B.

Results

Up-regulation of CPT1C by lentiviral transduction with shRNAmirs

We used lentiviral-delivered shRNAmirs directed against CPT1C and LMNA in different well-characterized human breast or brain cancer cell lines. A scrambled shRNAmir served as control for unspecific effects.

All cell lines transduced with the pseudotype viruses containing the shRNAmir constructs either directed against CPT1C or LMNA revealed an unexpected increase of CPT1C mRNA expression as assayed by quantitative RT-PCR. Similarly, there was also an unexpected increase in the cell lines transduced with the scrambled shRNA. In particular, transduction of T98G and MDA-MB-231 cells with the scrambled control led to an increase in CPT1C mRNA level by 473% and 326% respectively (Fig. 1A). Although the cells infected with the shRNAmirs directed against CPT1C showed a relatively reduced level of CPT1C mRNA it was still induced relative to the parental cell line.

To exclude that the transduction with the shRNAmir-containing virus was not causing a relative reduction of the internal control mRNA, GAPDH, we also examined the normalized mRNA expression of CPT1A and CPT1B. Neither CPT1A (Fig. 1B) nor CPT1B (Fig. 1C) expression was increased after transduction.

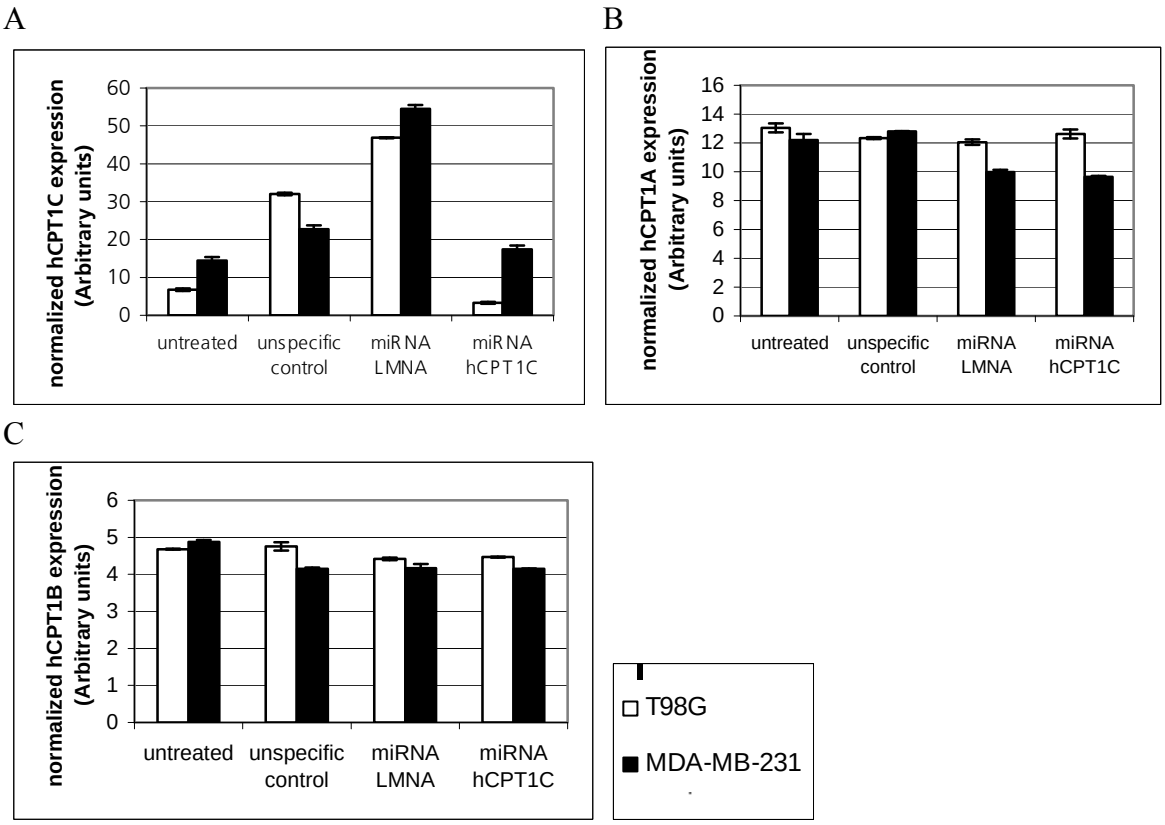

Figure 1: ***CPT1C mRNA level is elevated in response to transduction.***
Cells transduced with lentiviral based vectors containing different shRNAmir constructs. Normalized hCPT1C (A), hCPT1A (B), hCPT1B (C) mRNA expression in T98G (empty bars) and MDA-MB-231 (filled bars) cells.

CPT1C mRNA is not induced by stable transfection

In order to better understand what factors may be causing this unexpected induction of CPT1C, we used the shRNAmir plasmid without lentiviral delivery. In contrast to the cells which were successfully transduced with the replication-deficient viruses, cells genetically complemented with pcDNA6.2-GW/EMGFP-miR by transfection showed the expected loss of mRNA expression (Fig. 2).

CPT1A and CPT1B mRNA expression in the stable transfectants was used as an indicator for transfection-dependent changes in mRNA expression. MDA-MB-231 stable transfectants did not show any changes in CPT1A and CPT1B expression in the stable transfectants when compared to the untreated control (Fig. 2B, C). While CPT1A mRNA expression in the stable T98G transfectants was not changed upon transfection with the unspecific control shRNAmir or the LMNA shRNAmir, transfection with CPT1C shRNAmir led to an increase in CPT1A mRNA expression (Fig. 2B). CPT1A expression upon CPT1C knock down might be induced in order to compensate for the reduction in CPT1C mRNA level. CPT1B expression in contrast was reduced in all of the stable transfectants if compared to the untreated control independent of the transfected shRNAmir construct (Fig 2C).

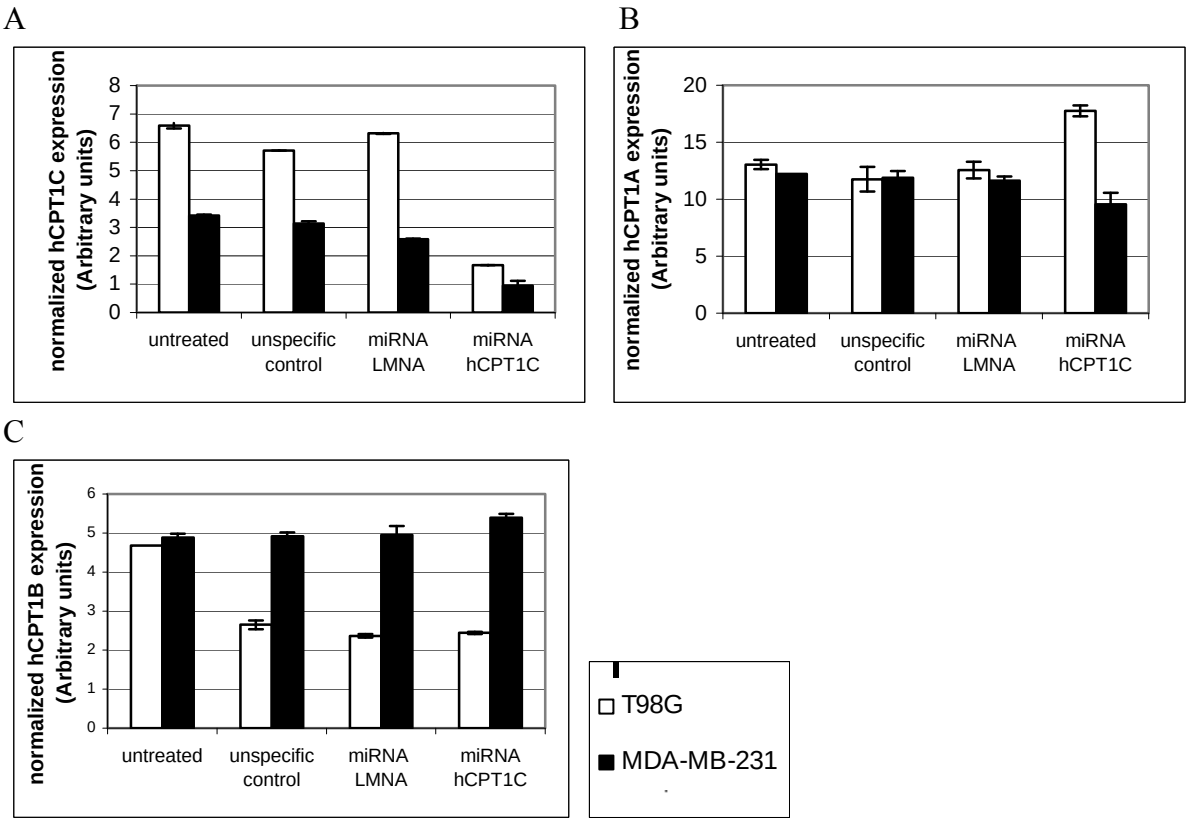

Figure 2: ***CPT1C mRNA is not upregulated in response to transfection.***
Transfection of cells with different shRNAmir constructs. Normalized hCPT1C (A), hCPT1A (B), hCPT1B (C) mRNA expression in T98G (empty bars) and MDA-MB-231 (filled bars) cells.

HIV1 infection induces CPT1C mRNA

In order to investigate whether HIV1 components of the lentiviral delivery system were responsible for CPT1C mRNA induction, we examined the impact of HIV infection on CPT1C mRNA. TZMBL cells were either mock infected or infected with HIV and examined for CPT1C mRNA expression. The HIV infected cells showed a 466% increase in CPT1C mRNA expression when compared to the Mock infected sample (Fig. 3A). As implicated by

the infection with the shRNAmir-containing virus, the mRNA expression of CPT1A (Fig. 3B) or CPT1B (Fig. 3C) was not affected by HIV infection.

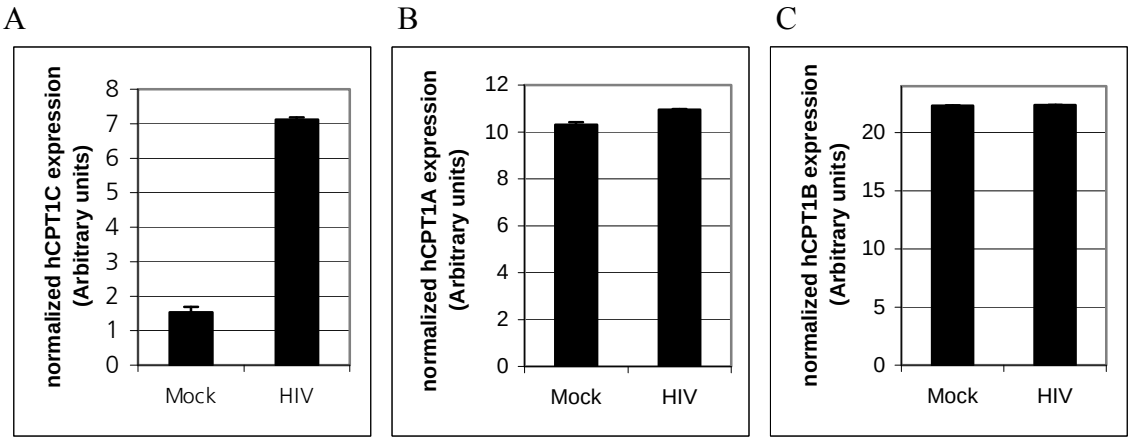

Figure 3: ***CPT1C is upregulated in response to HIV infection.***
Infection of TZMBL cells with HIV. Normalized hCPT1C (A), hCPT1A (B), hCPT1B (C) mRNA expression.

Discussion

Lentiviral systems are commonly used for the transfer of DNA into dividing as well as non-dividing mammalian cells.

All available viral systems contain only the parts of the viral genome that are absolutely necessary for efficient virus generation, target cell infection and integration of the gene of interest. The use of self-inactivating packaging vectors and specific cell lines for virus production are commonly used for the production of replication deficient viruses containing the gene of interest. Although these systems are well characterized under infection efficiency and biosafty criteria, the cellular side effects of those infections are not fully understood yet.

Independent of all the precautions undertaken during the generation of these lentiviral systems, the produced virus might nevertheless be able to change cell signaling upon infection.

The results presented here show that infection of established cancer cell lines with a lentiviral system used to transfer shRNAmir constructs results in the upregulation of CPT1C mRNA expression. This upregulation was not observed after chemical transfection of the shRNAmir constructs. Infection of TZMBL cells with HIV particles displayed an increase in CPT1C mRNA expression that was similar to the one observed upon infection with the shRNAmir-containing virus.

These results show that the undesired upregulation of CPT1C mRNA observed after viral transfer of shRNAmir constructs results from the virus infection and is not a consequence of the transferred shRNAmirs.

It is well established that viral proteins interact with host cell proteins to change cell metabolism and signaling, the effect observed in the presented experiments might therefore result from changes in cell signaling cascades during the viral infection.

The unspecific change in CPT1C mRNA level in response to virus infection might lead to phenotypic changes in the stable clones that are not induced by the transfer of the gene or shRNA of interest and which might lead to misinterpretations in phenotypic characterizations of stable knock down clones generated using lentiviral delivery systems.

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4 CPT1C: A Putative Regulator of Tumor Growth and Migration

I. Lohse*, K.Zaugg* et al.

* University Hospital Zürich, Department Radiation Oncology, Zürich, Switzerland

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CPT1C: A Putative Regulator of Tumor Growth and Migration

I. Lohse*, K.Zaugg*
* University Hospital Zürich, Department Radiation Oncology, Zürich, Switzerland

Abstract
All solid tumors independent of their origin contain areas of low oxygen. Hypoxic cells are highly aggressive and metastatic and mostly resistant to therapeutic approaches. Cell invasion and metastasis mark the most lethal phase of cancer and account for the majority of cancer deaths. Although very little is known about the molecular signaling pathways that trigger cancer metastasis, hypoxia is thought to activate signaling pathways that stimulate invasiveness of cancer cells.
The Carnitine Palmitoyltransferase 1c (CPT1C) has recently been identified as a p53-regulated brain-specific Carnitine Palmitoyltransferase (CPT) 1-family member that is upregulated in response to low oxygen availability. The role of CPT1C in brain, a tissue mainly utilizing glucose as a primary energy source, is still unsolved. Our previous data strongly suggest a role of CPT1C in the regulation of energy sensing and expenditure and furthermore implicate CPT1C in carcinogenesis.
In this study we examined the role of CPT1C in tumorigenesis in established human breast and brain cancer cell lines as well as in human pediatric brain tumor samples. The results presented here show that CPT1C expression level renders cell proliferation and invasion *in vivo* and *in vitro*. In addition, CPT1C expression level is regulated by hypoxia and HIF1α stabilization and correlates with tumor hypoxia in brain tumor samples.
Understanding of CPT1C function in carcinogenesis might give further insight in how cells are protected from hypoxic stress and may present a target through which to sensitize cells to hypoxia-induced cell death in cancer treatment.

Introduction
Metabolic stress, such as hypoxia, induces genetic and epigenetic selection for cancer cells to survive the hostile tumor environment. Although metabolic adaptation is prominently linked to a dysregulation of glycolysis, use of fatty acid oxidation (FAO) as an energy source has also been implicated in this process.
FAO is regulated at the step of fatty acid import into the mitochondria, which is controlled by activity of the CPT1 enzymes. Three CPT1 genes, demonstrating generalized tissue specificity, have been identified in mammals. The physiological roles of CPT1A, the prominent CPT1 gene in liver, and CPT1B, the prominent CPT1 gene in muscle, are well established primarily due to their role in pathogenicity (1). In contrast, the physiological role of CPT1C, the isoform primarily expressed in brain tissues, remains elusive.
The fact that CPT1C is mainly expressed in the CNS (2, 3), a tissue normally not using fatty acids as a major energy source, suggests a potentially unique function for CPT1C. Recent publications show that CPT1C expression in the brain is mainly restricted to the hypothalamic feeding centers, where lipid metabolism is believed to play a key role in regulating peripheral energy homeostasis. Results derived from studies using Cpt1c knock-out mice implicate CPT1C in the regulation of energy homeostasis and the control of food intake (3, 4). Although Cpt1c has been shown to be involved in hypothalamic energy sensing, its molecular function, regulation and signaling pathways still remain controversial (3, 4, 5, 6).
It has been shown that CPT1C mRNA expression is induced by hypoxia. In addition, cancer cell lines depleted of CPT1C display reduced cell proliferation and Xenograft tumor growth (7).
Analysis of CPT1C expression in lung cancer samples revealed that CPT1C is overexpressed in most of human lung tumor samples, suggesting an involvement of CPT1C in cancer. The

role of CPT1C in tumor formation and cancer progression nevertheless needs to be further elucidated.

The ability of tumor cells to invade distant tissue is a critical step in metastasis and the main cause of cancer-related death. This process involves the detachment of cells from the primary tumor, invasion through the basement membrane, intravasation into the blood stream and extravasation from the blood stream at a distant site where cancer cells proliferate and give rise to metastases (8, 9, 10, 11). It is well established that normal cells as well as tumors need oxygen to generate energy and most of the fundamental processes driving cell proliferation and survival. In tumor cells nevertheless, hypoxia is also strongly associated with tumor progression and metastasis (12, 13, 14, 15, 16). It has been hypothesized that low oxygen tension promotes metastasis by causing the cells to switch to anaerobic metabolism and promoting angiogenesis (17). This hypothesis has been strongly supported by studies showing that the Hypoxia Inducible Factors (HIFs) play critical roles during tumor cell metastasis by regulating energy metabolism, the induction of angiogenesis, cell detachment, invasion, and tumor cell seeding (13, 15, 17, 18). Yet, the signaling pathways and molecules involved in the hypoxia-dependent induction of tumor cell spreading mostly remain elusive.

Our study aimed to investigate a possible involvement of CPT1C in tumorigenesis and cancer progression as it was suggested by earlier studies (7). Here we show that CPT1C expression renders cell proliferation and migration in established brain and breast cancer cell lines. Cells depleted of CPT1C show a significant reduction of cell proliferation under hypoxic conditions and a reduced migration and invasion potential, while cells constitutively expressing CPT1C display a dramatic increase in cell proliferation as well as metastatic potential *in vitro* and *in vivo* under normal growth conditions. Furthermore, we demonstrate that CPT1C mRNA expression is induced by hypoxia and HIF1 stabilization and that CPT1C mRNA expression correlates with tumor hypoxia in pediatric brain tumor samples.

Although very little is known about the function of CPT1C, the fact that CPT1C is mainly expressed in the central nervous system (3, 4, 5, 6), a tissue normally not using fatty acids as a major energy source, suggests a potentially unique function for CPT1C. Understanding the involvement of CPT1C in carcinogenesis might not only be a worthwhile route for the treatment of hypoxic tumors, but will also give further insight into the role of CPT1C in normal brain tissue.

Material and Methods

Plasmid construction

The shRNA sequences directed against CPT1C and LMNA as well as an unspecific negative control shRNA were designed, synthesized and annealed to the miR RNAi sequence. The generated shRNA was inserted into the pcDNA6.2-GW/EMGFP-miR vector and the sequence was verified.

The CPT1C Ultimate ORF clone was purchased from Invitrogen. The ORF was transferred from the pENTR221 vector (Invitrogen) to the pcDNA-DEST40 vector for His-tagged or pcDNA-DEST47 vector for GFP-tagged protein expression using the LR Clonase reaction (Invitrogen) according to the manufacture's guidelines and the sequence was verified.

Cell culture

The human cell lines T98G, MCF7 and MDA-MB-231 were purchased from the American Type Culture Collection (ATCC). The cells were maintained in monolayer culture in DMEM supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin. All cells were grown in a humidified atmosphere of 5% CO_2 at 37°C.

For stable cell line generation cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's manual. Stable clones were selected for 14 days and single clones were established for the subsequent experiments.
For hypoxic conditions, the cells were incubated at 37°C with 5% CO_2, 94% N_2 and 0.2% O_2 in a hypoxic incubator (Scholzen AG).

Quantitative RTPCR Analysis
Total RNA was extracted from cells with the Trizol Plus Mini-to-Midi RNA extraction kit (Invitrogen) and cDNA was prepared using the Superscript III cells direct cDNA synthesis kit (Invitrogen) according to the manufacturer's manual. qRT PCR was performed using the SYBR Green qPCR Super-Mix UDG kit (Invitrogen). GAPDH was used as an internal reference. Relative RNA levels were calculated by the ΔΔCt method (19). The following primers were used: fwd 5'- ATG ACA TCA AGA AGG TGG TG -3' and rev 5'- CAT ACC AGG AAA TGA GCT TG -3' for GAPDH; fwd 5'- TGG AAC TCA GTG CCC CTG TG -3' and rev: 5'- GCA GGA AAC ACA CCG GTG AG -3' for CPT1C, fwd 5'- TTA AAG GGA AGC GGG TCG TTA -3' and rev 5'- TCC ATT GTC CAA GCA GAA TTT GA -3' for PGK, fwd 5'- GCA CAT AGG AGA GAT GAG CTT C -3' and rev 5'- CCA CAG GGA CGG GAT TTC TTG -3' for VEGF.

Western Blot
Total protein was extracted after irradiation and protein concentration was determined. 50µg per sample were separated by 8% SDS gel and transferred onto PVDF membrane (GE Healthcare). The membranes were blocked with 2% non-fat-dry milk and probed with the anti-protein antibodies (Calnexin, Catalase, PCNA [Cell Signalling Technology]; actin [SIGMA-Aldrich]; tubulin [SIGMA Aldrich], V5 [SIGMA-Aldrich]). The blots were further incubated with horse radish peroxidase (HRP)-labeled antibodies (GE Healthcare) and the specific complexes were detected using the ECL Western Blotting Detection Reagents (GE Healthcare).

Growth curve analysis
To assay cell growth, exponentially growing cells were seeded into 6-well plates (NUNC) and incubated in a humidified atmosphere at 37°C. Total cell number per well was examined by counting every 24h using a Neubauer Improved Hemacytometer. Cells were either grown under normal growth conditions (5% Co_2) or under hypoxic conditions (5% CO_2, 94% N_2 and 0.2% O_2) as indicated.

Boyden Chamber Assays
Cells were resuspended 100µl in DMEM containing 5% FBS and loaded onto the upper chamber of a transwell plate with a pore size of 8.0µm Polycarbonate Membrane (Costar). 600µl conditioned DMEM containing 10% FBS was placed in the lower well, and cells were incubated at 37°C in a 5% CO_2-supplied incubator for 16 h. For invasion assays, the Polycarbonate Membrane was overlaid with 50µl Matrigel Matrix (Basement Membrane) (BD) diluted 1:20 in DMEM. To analyze the migration, cells that had migrated to the lower surface of the membrane were stained with 0.09% crystal violet and counted under bright-field microscopy. In each experiment, the numbers of stained cells in three independent image fields of the same size were averaged and indicated as relative migration. All migration and invasion experiments were done in triplicates and repeated at least 2 times.

Xenograft Assay
MDA-MB-231 cells (5×10^6) were injected subcutaneously into BALB/c-nude mice (CAnN.Cg-Foxn1<nu>/Crl) (Charles River Laboratories). Tumor growth was assayed in 6

mice per group. Tumor size was measured every two days. Tumor volume was calculated according to the formula, width^2 × length × 0.5.

In vivo metastasis Assay

The MDA-MB-231 CPT1C gain-of-function clones that were used in the Xenograft assays were transfected with the SV40-pGL4.26 luciferase vector for *in vivo* imaging. Stable clones expressing CPT1C and Luciferase as well as the appropriate control clones were injected into the tail vein of BALB/c-nude mice (CAnN.Cg-Foxn1<nu>/Crl) (Charles River Laboratories) with 10^6 cell per mouse. A stock solution of D-Luciferin19 (Calliper Life Sciences) at 15 mg/ml was prepared according to the manufacturer's instructions. 10 µl/g of body weight were injected intraperitoneally (IP) 10-15 minutes before imaging with the IVIS Imaging System 200 Series (Calliper Life Sciences). Mice were anesthetized with 5% isofluorane in an anaesthetization chamber, reducing to 3% isofluorane as soon as excitation phase passed. The mice were fully anesthetized and then transferred from the chamber to the nose cones attached to the manifold in the imaging chamber and isofluorane supply was reduced to 2%. Camera settings were adjusted and series of five images were taken first from the breast of the mouse and second series of five images from the back. Pictures were taken every third day over a 30 day experimental period. Organs were extracted at the end of the experiment and fixed with formalin. Paraffin-embedded sections were stained for hematoxylin and eosin (H&E staining).

Electron Microscopy

MCF7 stable transfectants were seeded on glass cover slips, grown until confluency in a humidified atmosphere of 5% CO_2 at 37°C over night and fixed in 2,5% glutaraldehyde for 30min. Fix was replaced with 2% OsO_4 for 30min, and the culture was rinsed and then stained with uranyl acetate for over night. Samples were dehydrated in an ethanol series, infiltrated with Epon, and polymerized. The embedded cells were cut from the cover slip and sectioned for image production.

Statistical analysis

All data are presented as means ± standard deviation (SD). The data was analyzed using the statistical program SPSS version 16.0 (SPSS Inc.). Differences among the groups were compared by one-way analysis in combination with post hoc Scheffe test. Two-tailed values of $p<0.05$ were considered significant.

Results

CPT1C mRNA expression is induced by tumor hypoxia in a HIF1- dependent manner

It has been demonstrated earlier that CPT1C mRNA expression is upregulated in response to hypoxia (7).

CPT1C expression in established brain cancer cell lines was examined under normoxic and hypoxic conditions to investigate if hypoxia regulates CPT1C expression. T98G cells treated with hypoxia over 12h showed a significant 2 fold upregulation in CPT1C mRNA expression (Fig. 1A). The hypoxia target genes VEGF and PGK mRNA expression were used as controls (Fig. 1A). HIF1 is the major transcription factor for the induction of gene expression in response to hypoxia. To investigate whether HIF1 is also responsible for the transcriptional induction of CPT1C expression we examined the mRNA expression in response to DMOG treatment. Treatment with the chemical compound DMOG, a specific cell permeable HIF-α prolyl hydroxylase inhibitor, results in the stabilization of HIFα under normoxic conditions and thus induction of HIF1-dependent target genes. T98G cells treated with 1mM DMOG for 12h displayed a 1.3 fold induction in CPT1C expression (Fig. 1B). The HIF1 target genes

VEGF and PGK served as controls for HIF1-dependent induction and were upregulated expectantly in response to DMOG treatment.
Tumor hypoxia is a critical factor in tumor progression because of the induced epigenetic changes which result in the selection of hypoxia-resistant aggressive tumor cells (12, 15, 17).
In order to investigate whether CPT1C expression correlates with tumor hypoxia *in vivo,* CPT1C and PGK mRNA expression were examined in 54 pediatric brain tumor samples of different WHO grades (Fig. 1C). Statistical analysis of the results obtained showed a significant correlation between PGK mRNA expression which served as a marker for tumor hypoxia and CPT1C mRNA expression (p=0.013)

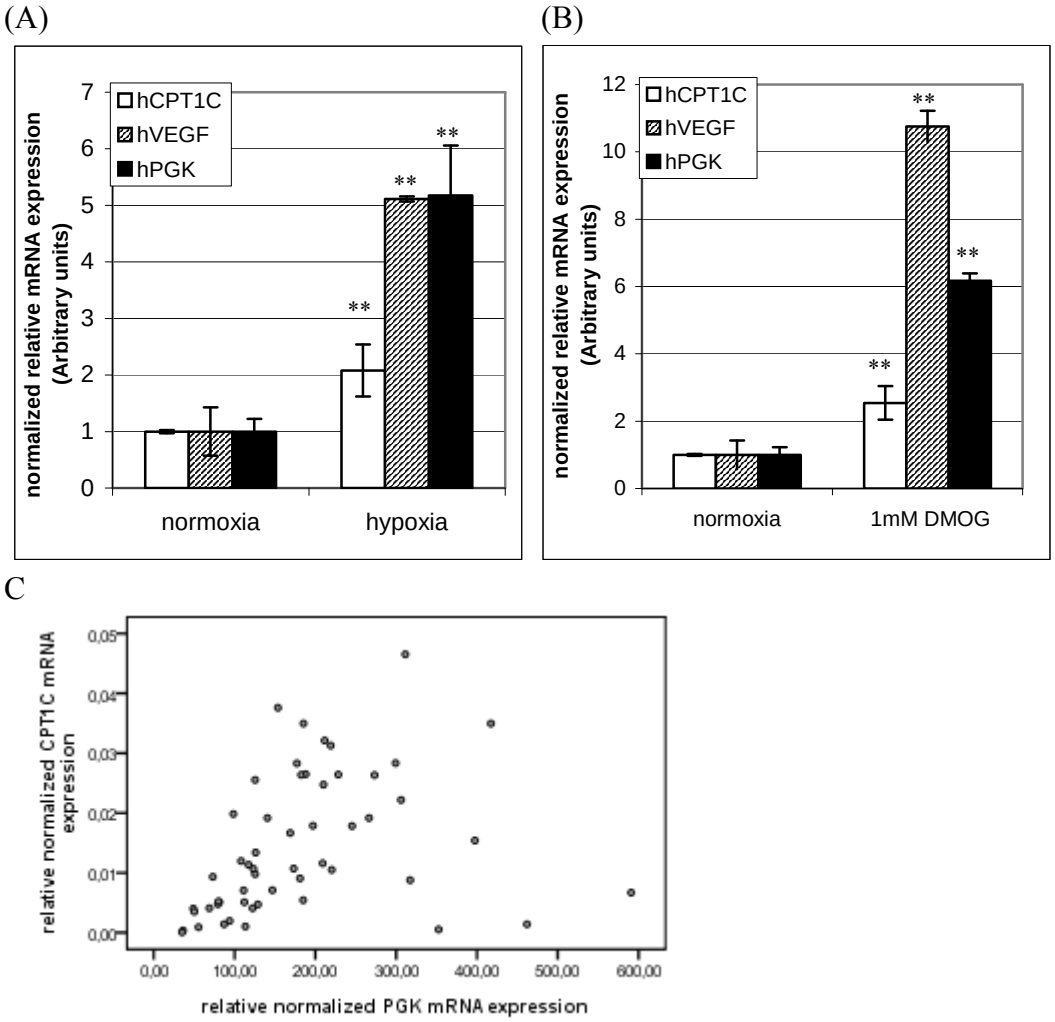


Figure 1: ***CPT1C expression is induced under hypoxic conditions***
qRTPCR analysis of T98G cells treated for 12h with (A) 0.2% O_2 and (B) 1mM DOMOG for 12h. Relative mRNA expression of CPT1C (empty bars), VEGF (striped bars) and PGK (filled bars) normalized to GAPDH. ** p≤ 0.001 (C) qRTPCR analysis of pediatric brain tumor samples. Relative expression of CPT1C and PGK normalized to GAPDH.

Constitutive expression of CPT1C leads to increased proliferation in vitro and in vivo
As shown previously, CPT1C is upregulated in lung tumor samples if compared to the matched control (7).
In order to examine the role of CPT1C in tumorigenesis (Fig. 1C), we generated CPT1C gain-of-function in different established cancer cell lines. CPT1C expression was examined by qRTPCR (data not shown) and Western Blot (Fig 2A), were V5-Tag was used for detection.

Growth curve analysis of T98G stable CPT1C gain-of-function transfectants displayed significantly reduced doubling times if compared to the control stable transfectants (Fig. 2B). Similar results were observed in MCF7 stable transfectants (data not shown). In contrast to the results obtained with T98G and MCF7 stable transfectants, MDA-MB-231 stable CPT1C gain-of-function transfectants showed no reduction in doubling time in the growth curve analysis (data not shown). But if injected subcutaneously into nude mice the stable transfectants showed a significant increase in tumor growth when compared to the vector control (Fig. 2C).

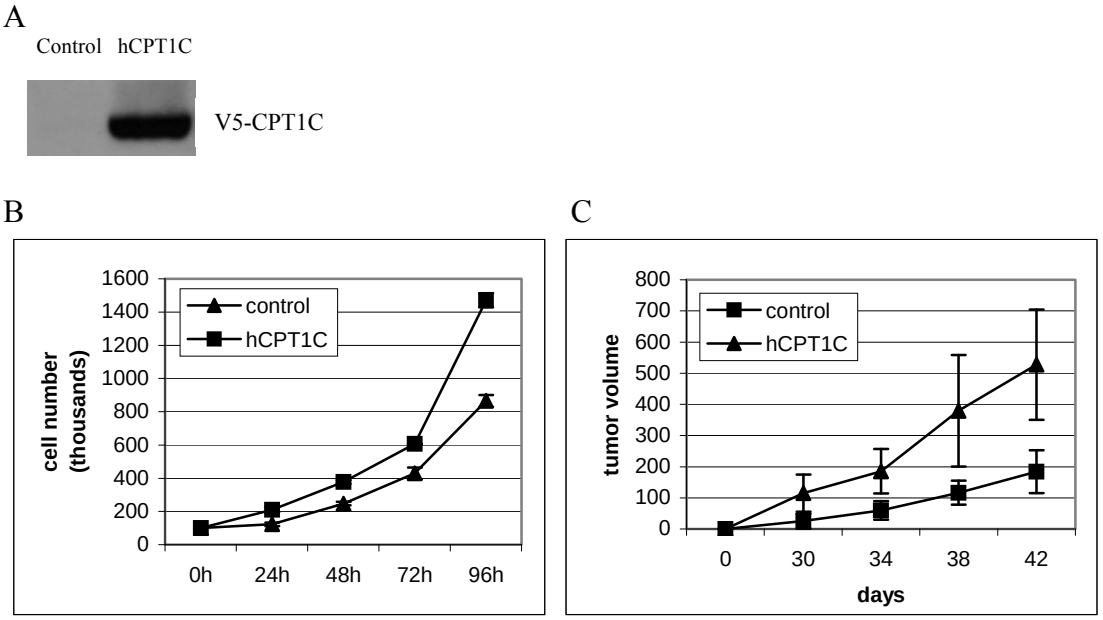
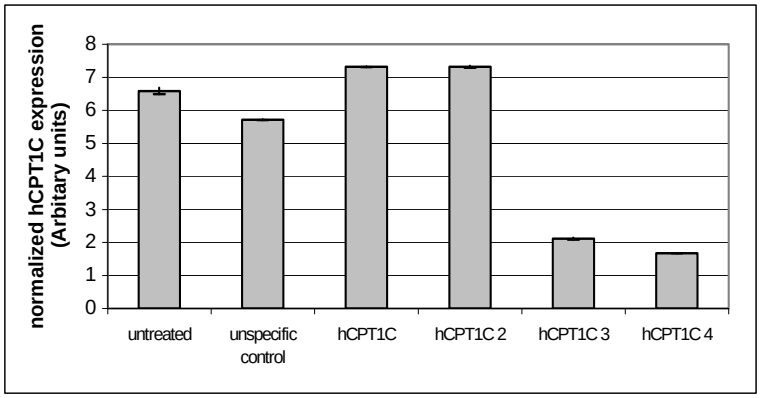


Figure 2: ***CPT1C gain-of-function induces cell proliferation and tumor growth***
(A) Western Blot of T98G stable gain-of-function transfectants for H5-tagged transgene expression. (B) Growth curve analysis of T98G cells constitutively expressing CPT1C. (C) Xenograft tumor growth assay of MDA-MB-231 gain-of-function stable transfectants.

CPT1C loss-of-function inhibits cell proliferation in response to hypoxia
CPT1C loss-of-function studies in established cancer cell lines showed that depletion of CPT1C results in reduced cell proliferation in response to hypoxia and Xenograft tumor growth (7).
To further investigate the phenotypic changes induced by CPT1C depletion, we generated CPT1C loss-of-function clones using four different shRNA sequences in T98G and MDA-MB-231 cells. CPT1C mRNA expression was examined by qRTPCR (Fig. 3A, data not shown) and the two clones with the strongest knockdown of CPT1C were used for subsequent analysis. Cell proliferation of these CPT1C loss-of-function transfectants was examined under normoxic and hypoxic conditions. CPT1C loss-of-function T98G clones showed a significant reduction in cell proliferation under hypoxic conditions if compared to the scrambled control clones (Fig. 3B). No reduction of cell proliferation was observed under normoxic conditions (data not shown). Similar results were obtained with the MDA-MB-231 stable CPT1C loss-of-function stable transfectants (data not shown).

A



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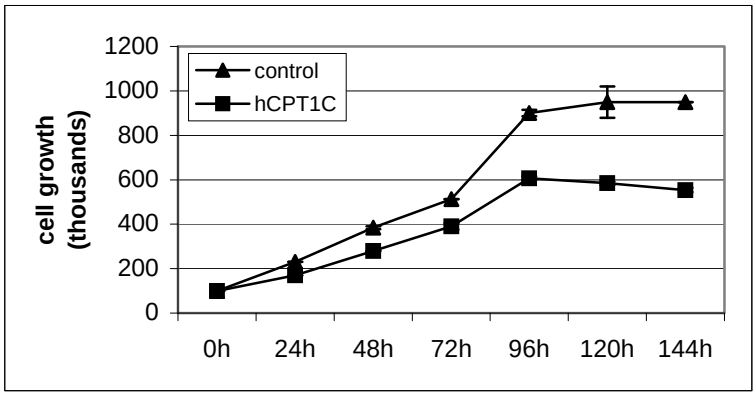


Figure 3: ***CPT1C loss-of-function reduces cell growth under hypoxic conditions***
(A) qRTPCR analysis of CPT1C expression normalized to GAPDH of T98G stable shRNAmir transfectants. (B) Growth curve analysis of T98G stable transfectants (control [triangles], CPT1C [squares] under hypoxic conditions.

CPT1C expression renders cancer cell migration and invasion in vitro
Intratumoral hypoxia correlates with poor clinical outcome in most solid tumors (12, 17). Hypoxic stress is a strong driving force during tumor progression and enhanced metastases formation by regulating energy metabolism (12, 15, 17). HIF1 signaling is thought to activate signaling pathways that stimulate invasiveness of cancer (13, 16). The molecular pathways involved, however, are not well understood (14, 15, 18). Our results showing regulation of CPT1C expression by hypoxia in a HIF-dependent manner as well as the proliferation phenotype prompted us to investigate a possible involvement of CPT1C in cancer cell migration and invasion.
The migration potential of the CPT1C gain- and loss-of-function cell lines was examined using Boyden chamber assays. All CPT1C gain-of-function stable transfectants, independent of the parental cell line and the expression level of CPT1C, showed a dramatic and highly significant increase in cell migration (Fig. 4A, data not shown) if compared to the stable control transfectants. CPT1C gain-of-function in T98G cells displayed a 3.5 fold increase in cell migration under normoxic conditions if compared to the control (Fig. 4A). Similarly increased cell migration potentials were found in all tested CPT1C stable transfectants (data not shown).
T98G stable loss-of-function cells on the other hand, showed a reduction of cell migration. In contrast to the hypoxia-dependent reduction in cell proliferation in response to CPT1C depletion, cell migration was reduced under both hypoxic and normoxic conditions. If compared to the control, T98G cells depleted of CPT1C showed a 1.7 fold reduction under

normoxic and 2.3 fold reduction under hypoxic conditions (Fig. 4B). Consistent with the results obtained with the gain-of-function stable transfectants, the MDA-MB-231 stable CPT1C loss-of-function transfectants showed a similar reduction in cell migration (data not shown).

Cell migration examined by Boyden chamber assays, is a potent indicator for the metastatic potential of tumor cells. Migration, however, is only one part of the metastatic process. To allow migration within the tissue, cancer cells need to digest the extracellular matrix in order to migrate towards the blood vessels.

Consistent with the results shown for cell migration, CPT1C expression also influences the cell invasion potential. T98G cell constitutively expressing CPT1C show a 7.1 fold increase in cell invasion (Fig. 4C) while CPT1C depletion decreases cell invasion by 2.3 fold under hypoxic and 3.6 fold under normoxic conditions (Fig. 4D). The same phenotype was also observed in all other cell lines tested (data not shown).

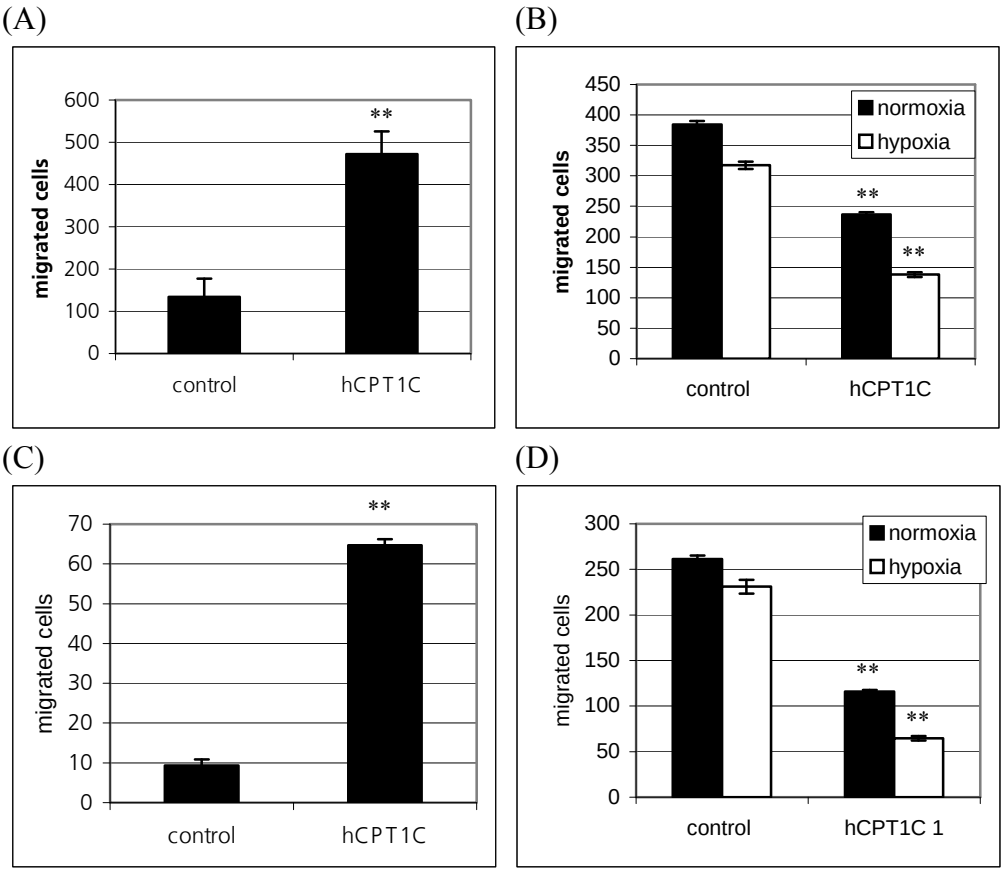


Figure 4: ***Cell migration and invasion potential is influenced by CPT1C expression***
Boyden chamber migration assays using T98G (A) CPT1C gain-of-function and (B) loss-of-function cells. Boyden chamber invasion assays of T98G (C) CPT1C gain-of-function and (D) loss-of-function cells. Cell migration and invasion was examined under normoxic (filled bars) and hypoxic (empty bars) conditions. ** $p \leq 0.001$

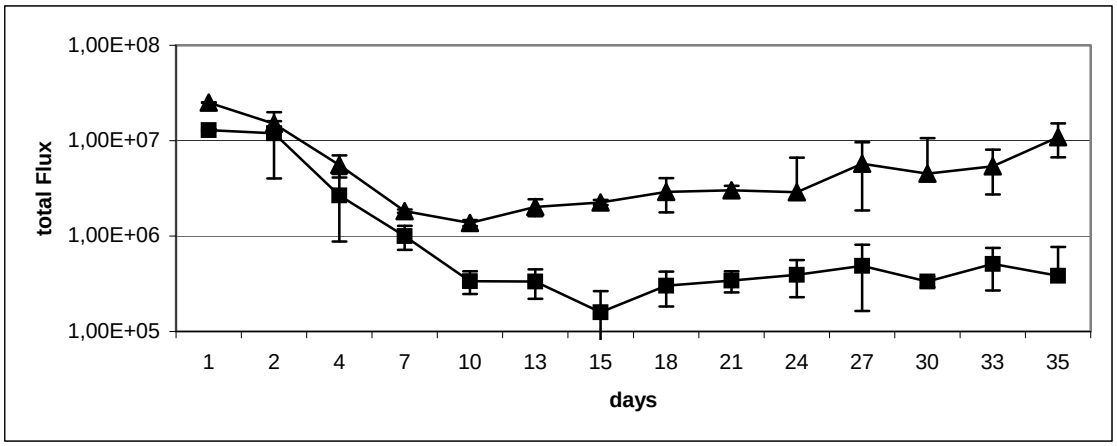
CPT1C expression renders cell invasion in vivo

While it is unclear how CPT1C may increase cell motility, the correlation between enhanced hypoxia tolerance and metastasis in the clinical disease (17) makes this a potentially worthwhile avenue for further investigations.

To examine whether the migratory phenotype observed *in vitro* is also observed *in vivo* we performed *in vivo* invasion assays. The MDA-MB-231 stable CPT1C gain-of-function

transfectants previously used in the *in vitro* assays were co-transfected with a constitutive firefly luciferase vector and injected into nude mice for the Xenograft migration assays. The chemiluminiscent signal detected after luciferin injection is used as a surrogate to estimate tumor growth.
One day after tail vein injection of the stable transfectants, we observed a strong signal in the lungs of all mice independent of the injected stable clone. Although this signal subsided until day 10, the signal derived from the lung significantly increased over the course of the experiment in the control mice (Fig. 5A). In the mice injected with the CPT1C stable transfectants in contrast, the signal subsided until day 15 and only showed a slight increase over the course of the experiment (Fig. 5A). No further signals above background derived from other organs than the lung were observed over the course of the experiment (Fig. 5A). Because of the rapid lung invasion observed in the control mice we had to sacrifice the mice.

(A)



(B)

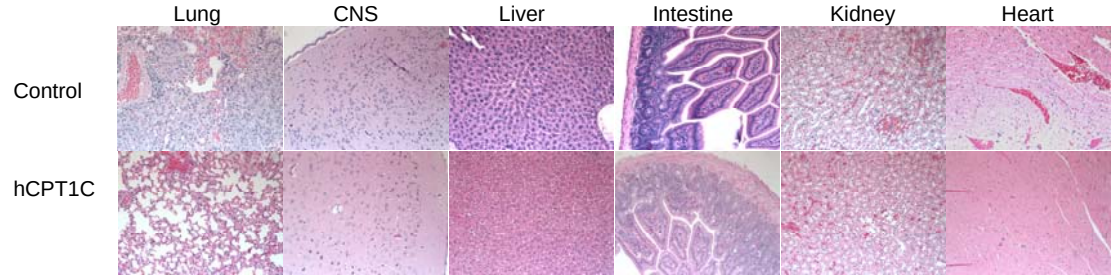


Figure 5: ***CPT1C gain-of-function increases migration in vivo***
Chemiluminescence optical tracking of the metastatic progression of MDA-MB-231 stable CPT1C transfectants. (A) Evaluation of chemiluminescence signals in the breast of mice injected with either stable control transfectants (triangles) (n=2) or stable CPT1C transfectants (squares) (n=2). (B) Histological confirmation of metastatic lesions formed by the MDA-MB-231 stable CPT1C transfectants injected s.c.. Metastatic lesions were identified by H&E staining. (preliminary results)

To confirm the results derived with the IVIS Imaging System, all organs were extracted and examined for metastatic lesions. Macroscopic examination revealed metastatic lesions of the lung in both the CPT1C gain-of-function and control mice, although the tumor invasion was more advanced in the mice injected with the vector control cells (Fig. 5B). No further lesions were observed during examination of brain, liver, heart, kidney and intestine of the control mice. Examination of the intestine of the CPT1C gain-of-function cells on the other hand showed an enlargement of the lymphatic plaques on the intestine (Fig. 5B).

H&E staining of the lung sections confirmed the metastatic invasion observed during animal imaging and macroscopic examination. As expected, the mice injected with the control vector clones displayed enhanced metastatic invasion of the lung if compared to the mice injected with the CPT1C gain-of-function clones and a nearly complete loss of normal lung tissue. None of the other organs extracted from the mice injected with either the control vector clones or the CPT1C gain-of-function clones displayed any metastatic lesion in the examined sections. Because only parts of the organs were examined, metastatic lesions might nevertheless be present in the unexamined parts. (preliminary data)

Constitutive CPT1C expression results in increased tubulin expression

It is well established that the cytoskeleton is the intracellular machinery responsible for changes in cell morphology and motility (20). Migration is driven by a continuous cycle of actin and tubulin polymerization and depolymerization (20, 21, 22). Cell migration induced by changes in the cytoskeleton can not only be induced by mutation of β-tubulin leading to polymerization and acetylation (20, 21), but also by up regulation of tubulin expression (23, 24, 25).

Although all of the generated CPT1C gain-of-function clones independent of the parental cell line displayed an increase in cell migration and invasion potential, we decided to use the MCF7 stable CPT1C transfectants and the corresponding control stable transfectants for the examination of the cytoskeleton. These clones had the highest CPT1C mRNA expression.

As suggested by the migration and invasion assays, the analysis of the images produced by electron microscopy revealed an increase in cytoskeleton stacks in cells expressing CPT1C which was not observed in cells transfected with the control vector (Fig. 6B). The cytoskeleton stacks were found in all cells examined and evenly distributed over the cells.

To validate the results obtained from the electron microscopy, we examined total tubulin expression by Western Blot analysis. As expected, the CPT1C stable transfectants showed a higher expression of tubulin if compared to the stable control transfectants (Fig. 6C). To exclude that the difference in tubulin expression was derived from a loading artifacts, total protein concentration was determined before loading and calnexin (Fig. 6C) among several other cellular proteins (data not shown) were used as loading control.

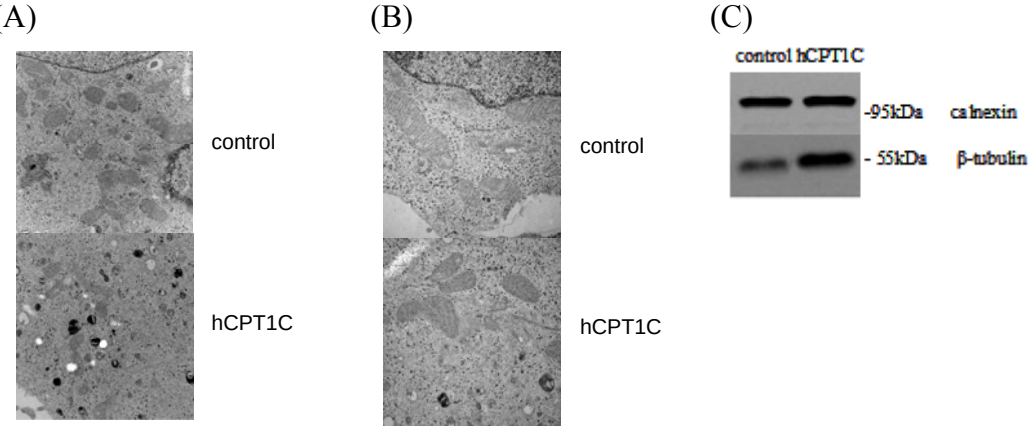


Figure 6: ***CPT1C gain-of-function renders cell morphology***

Electron microscope image of MCF7 stable transfectants with a 11500x magnification. Cells were examined for (A) inclusion bodies and (B) cytoskeleton. (C) Detection of calnexin and β-tubulin by Western Blot analysis of MCF7 cells stably transfected with CPT1C or the control vector.

Constitutive CPT1C expression results in the formation of intracellular vesicles

It has been published earlier that murine CPT1C knock out ES cells display morphological changes if examined by electron microscopy (26). These cells show a strong mitochondrial phenotype with enlarged mitochondria and a loss of cristae structure. Furthermore, these cells contain big lipid droplets.

In order to examine whether CPT1C loss- or gain-of-function in human cell lines will also affect cell morphology, we examined our stable transfectants by electron microscopy. In contrast to the results observed in the CPT1C knock-out ES cells, analysis of the T98G stable knock-down clones displayed no changes in cell morphology if compared to the scrambled control (data not shown). This might be due to the fact that our knock-down system only reduces CPT1C mRNA expression and that the remaining CPT1C protein is enough to maintain mitochondrial morphology.

MCF7 gain-of-function of CPT1C on the other hand showed an accumulation of intracellular vesicles that was not observed in the control cells (Fig. 6A). In order to determine the nature of these vesicles we examined the protein expression of several organelle markers by Western blot. None of the tested organelle markers was significantly increased in the gain-of-function cells if compared to the control cells (data not shown). Although the nature of the vesicles remains elusive they might serve as storage vesicles for over-expressed CPT1C protein. Further studies are necessary to elucidate the nature and function of these vesicles.

Discussion

It has been published previously that constitutive expression of CPT1C in cancer cell lines leads to increased FAO and ATP production while depletion of CPT1C has the reverse effect (7). Mouse studies show that CPT1C depletion results in a reduced body weight on a normal diet, but when fed a diet that is rich in fats, these mice demonstrate an increase in relative body weight concurrent with a reduction in both food intake and insulin resistance (3, 5). Nevertheless, the transferase activity and the substrate of CPT1C are still controversial in the literature (3, 4, 6). Previous results also showed that CPT1C expression is induced in lung tumors if compared to matched control tissue (7).

The results presented here show that depletion of CPT1C in cancer cell lines reduces cell proliferation under hypoxic conditions. Under normoxic conditions however, cell proliferation is not impacted by CPT1C depletion. Constitutive expression of CPT1C on the other hand results in reduced doubling times under normoxic conditions. Furthermore, CPT1C expression affects cell migration and invasion. CPT1C depletion resulted in cell migration potential in *in vitro* migration and invasion assays while constitutive expression of CPT1C had the opposite effect. In Xenograft metastasis assays in contrast, we could not detect an increase in metastasis formation in the mice injected with cells constitutively expressing CPT1C if compared to the control mice. The control mice showed a dramatic invasion of the lung tissues that forced us to sacrifice all mice. The lower amount of lung infiltration observed in the mice injected with cancer cells expressing CPT1C might result from an increased migration potential that allowed the CPT1C stable transfectants to leave the lung and invade other organs. Our experiments however were not able to give further insight in this effect, due to of the rapid lung invasion observed in the control mice that forced us to stop the experiment at an early time point. The induction of cell motility however represents only one aspect of tumor cell metastasis. Tumor cells that leave the primary tumor have to adapt to the blood flow and after the invasion of the distant tissue need to survive the new microenvironment. Our CPT1C gain-of-function stable transfectants might either not be able to overcome these restrictions or the survival time was too short to result in the formation of visible micrometastasis in the mice.

It is well established that tumor hypoxia induces an epigenetic selection for cancer cells that survive in the hostile tumor environment (12, 16, 18). Emerging evidence suggests that the

effect of hypoxia is partially controlled by HIF-mediated activation of pathways that enable tumor cells to survive or escape their oxygen-deficient environment. HIF promotes the selection and expansion of more aggressive clones of cancer cells by transcriptionally activating key regulators of metastasis (13, 14, 15) and therefore is believed to be the driving force of cancer metastasis (12, 14, 15, 16).

CPT1C expression is induced by hypoxic conditions and HIF1 stabilization. Additionally, CPT1C expression correlates with PGK, a marker of tumor hypoxia in pediatric brain tumors. Elevated CPT1C expression levels in cancer cells might therefore result in the activation of hypoxia-induced signaling pathways under normoxic conditions and thereby result in the observed phenotypes. The signaling pathways involved in the change of cell proliferation and migration however are not established yet. Further studies are necessary to elucidate the pathways induced by CPT1C in response to hypoxia and to understand the consequences of elevated CPT1C expression levels in human tumors.

Tumor hypoxia is a major therapeutic concern because it reduces the effectiveness of radio- and chemotherapy and promotes metastasis. While therapeutic strategies that capitalize on chronic hypoxia sensitivity are available, no cellular targets have yet been characterized that induce cellular sensitivity to hypoxia. Understanding how CPT1C expression influences cancer cells might give new insight for the prediction of therapy efficiency and furthermore suggest new therapeutic approaches for the therapy of otherwise treatment-resistant tumors.

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5 Discussion

CPT1C is the brain specific isoform of the CPT1 family. The CPT1 family of proteins has been shown to represent the limiting step of β-Oxidation by regulating the entry of LCFAs into the mitochondria. The fact that CPT1C is highly expressed in brain regions involved in the regulation of peripheral energy expenditure (48, 64, 116, 176, 189) suggests a potentially unique function for CPT1C.
CPT1C expression is found in neurons throughout the entire brain but shows the highest level in neural feeding centers within the hypothalamus. CPT1C expression is also enriched in amygdala and hippocampus, brain regions that are involved in food intake, reward and memory (147). Although the anatomical localization of CPT1C has been established by different studies, the subcellular localization is still controversial in the literature. Different studies have localized CPT1C to either the mitochondria or the endoplasmatic reticulum. Both organelles have been shown to be involved in the oxidation of FA. While the endoplasmatic reticulum is only involved in the oxidation of very LCFAs, the mitochondrial β-oxidation is used to degrade long- as well as short-chain FAs.
Cpt1c knock-out mice independent of the genetic background are viable and fertile and in a complex metabolic phenotype. These mice ingest less food and have a lower body weight than wild type (wt) littermates (116, 176, 189). When fed a high fat diet the Cpt1c knock-out mice have a markedly increased susceptibility to weight gain which can be reversed by the extopic expression of CPT1C in hypothalamic feeding centers (116, 176, 189). Additionally, Cpt1c knock-out mice display a severe insulin resistance in response to high fat diet (116, 176, 189). Furthermore, fasting conditions reduce whole body β-Oxidation of Cpt1c knock-out mice if compared to wt littermates (116, 176, 189).
The expression of CPT1C in the hypothalamic feeding centers and the metabolic phenotype observed in the knock-out mouse suggest for a regulatory rather than a metabolic role of CPT1C.
Hypothalamic feeding centers contain nutrient-sensitive neurons which have been shown to regulate the desire for food intake and satiety (29, 49, 52, 63, 82, 93, 107, 108, 117, 165) in response to specific stimuli. This requires the presence of molecular sensors that detect changes in whole body energy expenditure and trigger adaptive responses. Hypothalamic AMPK activity is regulated in response to cellular energy level, circulating hormones and nutritional cues (29, 44, 52, 63, 93, 117, 165, 224) and is used to sense changes in the whole body nutrition status. The modulation of AMPK activity in response to these factors initiates signaling pathways leading to changes in feeding behavior (140, 165). The molecular mechanism(s) by which hypothalamic AMPK regulates the desire for food intake however are still poorly understood. Plasma FA levels are detected by specialized hypothalamic neurons which integrate this information in the regulation of feeding behavior.
A database search on NCBI revealed that the human CPT1C genes gives rise to two transcript variants that differ in length and exon content. The existence of these two transcript variants might explain the contradictory results obtained by different studies concerning the intracellular localization and transferase activity (48, 116, 170). It is well possible that these isoforms differ in the subcelluar localization and affinity towards their substrate palmitate and serve as regulators of different downstream pathways. Further studies which aim to understand the expression profile of the transcript variants might solve the controversy about CPT1C localization and function.
Both mRNAs contain a conserved uORF upstream of the CPT1C-coding mORF. CPT1C mRNA sequence analysis revealed that the presence of an uORF is conserved in CPT1C mRNAs of different species but not within the CPT1 family of proteins. Because eukaryotic ribosomes generally only initiate once per mRNA (38, 158, 182), the presence of an uORF

normally inhibits the translation of the mORF and may lead to mRNA decay (3, 8, 35, 37, 152, 166).
Using an *in vitro* luciferase reporter assay we showed that the translation of the downstream mORF is indeed repressed by the presence of the uORF. In response to glucose deprivation the mORF was released from the translational repression resulting in an enhanced expression of the reporter gene. This translational derepression of the mORF was not observed in response to serum starvation. Because AMPK has been shown to change gene expression in response to changes in energy availability, we also investigated the impact of AMPK activity on the translational repression of CPT1C by the uORF. AMPK inhibition by pharmacological compounds such as Metformin and Compound C leads to the derepression of the mORF independent of glucose availability, while treatment with the AMPK agonist AICAR results in repression of the mORF even during glucose starvation. In addition, the mORF is also derepressed in response to Palmitate-BSA feeding. Treatment with Oleate-BSA or Octanate-BSA however did not result in derepression of the mORF. This suggests that the uORF regulates the mORF expression in response to a specific set of stimuli rather than mediating a general stress response. Surprisingly, treatment with the mTOR inhibitor Rapamycin did not affect the uORF mediated repression of the mORF. The specificity of the AMPK and mTOR inhibitors and the AMPK agonist AICAR was established by earlier studies (44, 52, 82, 93, 107, 131, 146).
Overall, these results suggest that CPT1C translation is regulated by the presence of the uORF and that this regulation is impacted by cellular energy availability and AMPK activity. Combined with the fact that CPT1C is expressed in neurons of hypothalamic nuclei (48, 147) and that the Cpt1c gene-deficient mouse demonstrates a metabolic phenotype (116, 176, 189), these results support the evidence that CPT1C gene expression may be an important AMPK effector in regulating satiety. The detailed mechanism through which CPT1C may regulate peripheral energy sensing however is still unclear.
The posttranscriptional regulation of CPT1C expression might be used to regulate the activation of downstream signaling pathways in response to energy availability. The membrane localization provides the possibility that changes in CPT1C expression impact ion channel functions, which are critical in neuronal signaling. Regardless, the post-transcriptional regulation suggests that CPT1C gene expression must rapidly change to perform its role.
It has been previously shown that AMPK can inhibit translation by mTOR (222). Our finding that Rapamycin does not induce derepression suggests that AMPK is functioning via a different and novel mechanism. Further biochemical studies will be necessary to elucidate the factors through which AMPK activation may control this translational derepression. It is also unclear whether AMPK and Palmitate-BSA may be functioning through a common mechanism. The previous finding that Palmitate may regulate hypothalamic signaling of satiety through inhibition of insulin signaling (25) may be an important clue to further elucidate the specific effectors between cellular energy availability and CPT1C 5'UTR derepression. Further studies are necessary to examine the importance of this regulatory mechanism in the hypothalamic regulation of satiety under physiological conditions.
In additions to its role in normal brain, the p53-target CPT1C has also been hypothesized to play a role in cancer. The involvement of CPT1C in the hypothalamic regulation of energy sensing and expenditure combined with the finding that CPT1C is upregulated in lung tumor samples strongly suggest for an involvement of CPT1C in cancer development.
Obesity, virus infections and genetic predisposition are established to be important factors in the development of cancers (WHO). Though the means by which obesity influences cancer development and progression are not fully understood.
Initially we attempted to generate stable CPT1C loss-of-function clones in various established brain- and breast cancer cell lines using a HIV-based lentiviral system. The use of lentiviral

systems for the transfer of microRNA-adapted shRNAs (shRNAmirs) allows the rapid and efficient generation of stable loss-of-function clones in cell lines. All cell lines transduced with the pseudotype virus containing a control shRNAmir or shRNAmirs directed against CPT1C or LMNA revealed a dramatic increase in CPT1C expression that was not observed after chemical transfection of the same shRNAmirs. A similar upregulation was observed in cells infected with HIV-1. Neither of the other CPT1 family members was induced upon transduction with the pseudotype virus or infection with HIV-1. These results show that HIV infection specifically upregulates CPT1C mRNA expression through a yet unknown pathway.
The viral components of the lentiviral system might activate pathways that are also induced during the virus infection-induced metabolic remodeling. Interestingly, HIV patients have been shown to commonly suffer from wasting diseases that are still poorly understood (168, 188). Induction of CPT1C expression in response to virus infection might be used to achieve the observed metabolic changes in order to allow the efficient production of new virus.
Further studies are necessary to establish the molecular mechanism by which HIV infection activates CPT1C expression and to investigate if upregulation of CPT1C expression is involved in the manifestation of the metabolic symptoms in HIV-infected patients.
It has been shown earlier that CPT1C expression is induced by hypoxia which is supported by our data (236). Our results furthermore show that CPT1C expression is upregulated in response to HIF-1 stabilization. Treatment of different established brain cancer cell lines with the chemical compound DMOG, a competitive inhibitor of HIF-PH that acts to stabilize HIF-1α expression at normal oxygen tensions, results in the induction of CPT1C mRNA expression. Additionally, CPT1C mRNA expression correlates with the mRNA expression of the HIF-1 target gene PGK in pediatric brain tumor samples. HIF target genes have been shown to regulate a number of cellular pathways such as metabolic adaptation, proliferation and metastasis.
To elucidate the role of CPT1C in cancer progression we examined the consequences of CPT1C gain-of-function and loss-of-function in human breast and brain cancer cell lines. An earlier study showed that CPT1C depletion results in decreased proliferation rates if the cells are exposed to hypoxia (236). This phenotype was also observed in our stable transfectants. CPT1C depletion in brain and breast cancer cell lines displayed no proliferative phenotype under normoxic conditions. If the cells were treated with hypoxia, CPT1C stable loss-of-function transfectants revealed a significant decrease of cell proliferation.
Our results furthermore showed that CPT1C gain-of-function leads to a significant increase in cell proliferation. Interestingly, this induction of cell proliferation was observed under normoxic conditions. All stable CPT1C gain-of-function transfectants except for the clones derived from MDA-MB-231 cells displayed significantly reduced doubling times if compared to the control stable transfectants. But if injected subcutaneously into nude mice, the MDA-MB-231 stable transfectants showed a significant increase in tumor growth when compared to the vector control.
Continuous proliferation imposes a number of problems because of an increased metabolic demand and microenvironmental changes in response to an increasing cell mass. Thus, signals that simulate cell proliferation must also participate in the reorganization of cell metabolism to allow quiescent cells to start proliferation. The metabolic adaptation of cancer cells is mainly believed to function through adaptations of the glycolytic pathway. Recent studies nevertheless show that some fatty acids play a role in enhancing cell proliferation and represent an important energy source. Tumor hypoxia induced by rapid tumor cell growth is present in all solid tumors and represents an important threat on tumor cell survival. In additions, hypoxia in tumors triggers the selection of more aggressive clones that are able to adapt to the hostile microenvironment. In mammalian cells, the cellular response is mainly mediated by the HIF transcription factor complex which regulates a wide number of target

genes involved in angiogenesis, metabolic adaptation and metastasis and thereby orchestrates the tumor phenotype.

Previous studies proposed that CPT1C has a regulatory function rather that playing a direct role in the import of LCFAs into the mitochondria. This is supported by the fact that CPT1C is highly expressed in the hypothalamic feeding centers where it is believed to be involved in the regulation of peripheral energy homeostasis (116, 176, 189). The function of CPT1C in cancer cells might thus be similar to its function in normal tissue. If induced by HIF stabilization in response to tumor hypoxia, CPT1C might sense energy availability and integrate this information by activation of downstream signaling pathways. Activation of these pathways might induce changes in cell metabolism, for example by activating CPT1A and CPT1B which results in increased β-oxidation rates, which subsequently regulate cell proliferation. Reduced CPT1C expression under hypoxic conditions might therefore result a decreased activation of the downstream pathways leading to a reduction in cell proliferation. Constitutive expression of CPT1C in contrast may lead to the activation of hypoxia-dependent signaling cascades in the absence of HIF stabilization which results in the induction of cell proliferation. Although the metabolic phenotype could also be explained by a direct function of CPT1C in the import of LCFAs into the mitochondria, the localization of CPT1C expression to brain regions involved in feeding behavior as well as its low expression level favor a regulatory function of CPT1C.

As a next step, we examined if changes in CPT1C expression also affect cell migration and invasion. Tumor metastasis is also regulated by HIF stabilization and is believed to function as survival mechanism for tumor cells.

Stable CPT1C loss-of-function transfectants displayed a reduction of cell migration and invasion in *in vitro* Boyden chamber assay. Surprisingly, the reduction of cell migration and invasion potential was observed under normoxic as well as hypoxic conditions. Constitutive expression of CPT1C, in contrast, resulted in the induction of cell migration and invasion in the Boyden chamber assay.

However, mice injected with CPT1C stable gain-of-function transfectants co-expressing Luciferase, showed a reduced lung-derived signal if compared to the control mice. The chemiluminiscent signal correlates with tumor growth in the corresponding organ. Mice injected with stable control transfectants showed a dramatic invasion of the lung tissue. No further signal was observed in any of the mice over the course of the experiment.

To examine whether the mice contained any micrometastasis that were too small to evoke a luminescent signal above the background, all organs were extracted, sectioned and stained. These stainings showed no micrometastasis formation in any of the examined organs except the lung. It might nevertheless be possible that the extracted organs contained micrometastasis in parts which were not stained and examined by microscopy. (preliminary results)

The lower amount of lung infiltration observed in the CPT1C-injected mice might result from an increased migration potential that allowed CPT1C stable transfectants to leave the lung and invade other organs. Our experiments however were not able to elucidate this effect because of the rapid lung invasion observed in the control mice that forced us to stop the experiment. Further experiments are necessary to elucidate the *in vivo* migratory phenotype. The induction of cell motility represents only one aspect of tumor cell metastasis. Tumor cells that leave the primary tumor have to adapt to the blood flow and after invasion of the distant tissue need to survive the new microenvironment. These aspects of the metastatic cascade are regulated by independent pathways. Our stable transfectants expressing CPT1C might either not be able to overcome these restrictions or the experiment was not long enough to result in the formation of visible micrometastasis in the mice. It is possible that constitutive CPT1C expression results in the induction of pathways that regulate cell motility without affecting pathways that regulate other aspects of metastasis.

Although HIF has been shown to regulate both cell proliferation and metastasis, the underlying pathways are different. Our results show that cell proliferation and invasion are influenced by CPT1C expression. What remains unclear are the mechanisms underlying these phenotypic changes. The induction of CPT1C in response to HIF stabilization might regulate the activity of a number of different pathways leading to proliferation and migration. But it is also possible that CPT1C only activates pathways that modulate cell metabolism. The observed phenotypes may therefore only be side effects induced by the metabolic adaptation to hypoxia and the subsequent increase in ATP production.
Further studies are needed to elucidate the pathways that are regulated in response to changes in CPT1C expression. These studies might elucidate if CPT1C regulates cell proliferation and migration via the induction of two different pathways, or if the same signaling cascade activates both processes.
We also examined the effect of CPT1C gain-of-function and loss-of-function on cell morphology. A previous study revealed that CPT1C depleted murine ES cells display a severe change in mitochondrial morphology and the accumulation of lipid droplets in the cytoplasm (236). The mitochondrial phenotype is characterized by swollen mitochondria that display an abnormal internal membrane structure and a loss of cristae structure. This phenotype was not observed in our T98G CPT1C loss-of-function stable transfectants. The knock-down achieved by the introduction of siRNAs however is only partial, while the knock-out achieved by genetic approaches for the generation of knock-out mice is complete. The remaining CPT1C protein might therefore be sufficient to maintain the mitochondrial integrity.
In contrast to CPT1C loss-of-function stable transfectants, stable gain-of-function of CPT1C in MCF7 cells displayed two distinct morphological changes if compared to the control clones. Examination of the CPT1C gain-of-function cells by electron microscopy revealed that these cells contain vesicles of unknown origin. CPT1C contains two intermembrane domains that tightly associate the protein to the target membrane. CPT1C expressed above physiological level needs to be localized to a cellular membrane. These intracellular vesicles might function as storage vesicle for the excess of CPT1C protein that due to space limitations can not be localized to the mitochondria and/or endoplasmatic reticulum.
In addition, these cells also display an increase in cytoskeletal stacks. This observation was confirmed by Western Blot analysis showing an increase in tubulin expression in the CPT1C stable gain-of-function transfectants if compared to the control clones. It is well established that the cytoskeleton is the intracellular machinery responsible for changes in cell morphology and motility. Migration is driven by a continuous cycle of actin and tubulin polymerization and depolymerization (76, 121, 144, 173, 190, 201). The observed increase in tubulin expression results from the induction of metastasis and might not be directly induced by the constitutive expression of CPT1C.
Elucidating the mechanism (s) by which CPT1C influences the sensitivity to hypoxia, cell proliferation and cell motility may provide a novel approach to improve prognosis of treatment-resistant cancers and present a target through which to sensitize hypoxic cells to cancer treatment.

Overall, the results presented here give further insight into the function of CPT1C in normal and cancer cells.
Our data show that CPT1C expression is regulated at the step of translational initiation in response to cellular energy availability. Furthermore, our results demonstrate that CPT1C expression levels influences cell motility and proliferation.
Although the results presented here add further insight in to the function of CPT1C in normal tissue and in carcinogenesis, the studies revealed a number of new and yet unsolved questions at the same time.

6 References

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7 Appendix

7.1 Effect of high dose per pulse flattening filter free beams on cancer cell survival

Lohse I*, Lang S*, Hrbacek J*, Klöck S*, Lütolf U M*, Zaugg K*
* University Hospital Zürich, Department of Radiation Oncology, Zürich, Switzerland

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Effect of high dose per pulse flattening filter free beams on cancer cell survival

Lohse I*, Lang S*, Hrbacek J*, Klöck S*, Lütolf U M*, Zaugg K*
* University Hospital Zürich, Department of Radiation Oncology, Zürich, Switzerland

Abstract
Radiotherapy represents one of the most effective tools in the treatment of human cancers. The development of new linear accelerators' like the TrueBeam allow the increase in average dose resulting from the removal of the flattening filter. The use of flattening filter free (FFF) beams reduces patient treatment time by increasing dose-per-pulse as well as dose-rate and might therefore achieve higher treatment efficacy.
Ionizing radiation (IR) induces a number of cell signaling pathways that include not only DNA damage recognition and repair pathways but also signaling cascades induced by unfolded proteins and organelle damage. Although the cellular response to IR is well established, the impact of dose-rate and unflattened beams on cancer cell survival has not been fully investigated yet.
The results presented here show that clonogenic survival is reduced upon treatment with ionizing radiation using high dose-rates. Furthermore, we show that the use of unflattened beams reduces cell survival after irradiation through an increase of dose per pulse in a total dose-dependent manner.Understanding the mechanisms by which dose-rate and dose-per-pulse influence cancer cell survival might propose new approaches for the therapy of treatment-resistant tumors.

Introduction
Recently, the TrueBeam linear accelerator (Varian Medical Systems), which allows photon treatments without the flattening filter in place, was released for clinical use. The removal of the flattening filter leads to an increase in the average dose-rate, which allows a significantly faster delivery of the dose to the patient. The beam pulse characteristic of FFF beams is different from the one of classical flattened beams. An FFF beam running at the same average dose rate as the flattened beam has an increased dose per pulse (instantaneous dose rate) and a lower pulse repetition frequency. This makes it possible to investigate the tumor cell survival under an increased instantaneous dose rate. Whilst there is evidence in the literature showing that prolonged treatment times lead to an increase in the tumor cell survival (1, 2), very little is known about tumor cell survival for increased dose-rates.
The cellular response to IR, as it is applied by the linear accelerators used for patient treatment, involves the activation of multiple signaling pathways (2). It has been well established that cells exposed to IR show DNA damage, increase mutation rates, chromosome aberrations and finally apoptosis. DNA damage induced by IR arises not only from direct effects but is also induced by reactive oxygen species derived from water hydrolysis. Because of their impact on genome stability and proliferation, DNA double strand breaks (DSB) are the most damaging DNA lesion after radiation (4, 5). Once detected, DSB are repaired by two specialized pathways, namely homologous recombination or non-homologous end joining (6).
Although DNA damage represents the most severe threat to cell survival after IR, other cellular structures like proteins, lipids or organelles are also affected (4, 5, 7, 8, 9, 10).
Additionally cells are also impacted by so-called bystander effects which include reduced clonogenic survival, altered gene expression and apoptosis due to signals transmitted via gap-junctions or factor secreted to the medium by damaged cells. Bystander effects induced by the irradiated cells lead to the activation of signaling cascades that affect the response of the irradiated cell population (11).
Glioblastoma multiformae are among the most malignant human cancers. IR represents one of the most important therapies in brain cancer treatment because of its independence of the

blood-brain-barrier which limits substance application. Glioblastoma usually have a poor prognosis because of their intrinsic resistance to radio-and chemotherapy (12, 13, 14, 15).
Here we report that the induction of cell death in brain cancer cells in response to irradiation not only depend on the total dose delivered per treatment but also on dose-rate and dose-per-pulse. Irradiation of T98G and U87-MG cells with different dose rates revealed that an increase in dose-rate leads to a reduction in clonogenic survival. In addition, we were able to show that independent of the dose rate, FFF beams are more efficient in inducing cell death than the more commonly used flattened beams because of the higher dose that is applied per single pulse.
The results shown here might present an opportunity to investigate the effect of dose-rate and dose-per-pulse as a potential therapeutic option in radiotherapy-resistant cancers.

Material and Methods

Cell culture

The human glioblastoma cell lines T98G, expressing mutated p53, and U87-MG, expressing functional p53, were purchased from the American Type Culture Collection (ATCC). The cells were maintained in monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) containing supplemented with 10% FBS, 1% L-Glutamine and 1% Penicillin/Streptomycin. All cells were grown in a humidified atmosphere of 5% CO_2 at 37°C.

Irradiation

Pulsed photon beams of the TrueBeam linear accelerator (Varian Medical Systems) using the nominal energy 10 MV, with the flattening filter in place (further X10) and without the flattening filter (further X10FFF) were used in this study. Characteristics of the two beams are in detail summarized (16, 17, 18, 19). In short, the absence of the flattening filter leads to a decrease of a beam's mean energy and to an increase of dose delivered per pulse (DPP) of radiation. Each beam can be produced at different dose rate (DR) which is achieved by modifying the pulse repetition frequency (PRF). The change of dose rate does not influence the dose per pulse (Fig. 1). Table 1 summarizes the quality index (Q-index), which is an indicator for the mean energy, dose per pulse, and pulse repetition frequency for beams and dose rates used in our investigation.

Table 1: ***Beam characteristics***

Energy	Q-index	DPP	DR	PRF
		(MU/puls)	(MU/min)	(Hz)
X10FFF	0.691	0.13	2400	360
			400	60
X10	0.735	0.028	600	360
			400	240
			20	12

Irradiation of samples was performed in the reference geometry, i.e. source-to-surface distance of 100 cm, field size 10x10 cm^2, at the depth of maximum. The samples were placed on 5 cm of water equivalent RW3 plates (PTW). The build-up for every beam was provided by RW3 plates of appropriate thickness placed on top of the samples. In the reference conditions, 100 MU corresponds to 100 cGy, hence dose per pulse expressed in the units of MU/pulse or cGy/pulse is the same.
Additionally an experiment was designed comparing X10FFF beam with X10 beam for the same DPP and the same PRF. The irradiation with the X10 beam, DR 400 MU/min was done

as described above. To achieve the same conditions for the X10FFF beam, it had to be attenuated by a factor of four. This was achieved by 30.5 cm of RW3 plates. To deliver the same dose the number of MUs was increased by a factor of four and to radiate with the same PRF a DR of 1600MU/min was used.
A diameter of a sample plate is 30mm. Three samples for the flattened beams and only one sample for the unflattened beams were irradiated at a time in order to deliver homogeneous dose to the samples. An analysis of dose profiles of corresponding beams shows that dose within a sample should not vary more than ±2%.
An independent verification of absolute dose for the irradiation setup was performed using gafchromic film (EBT2, ISP).

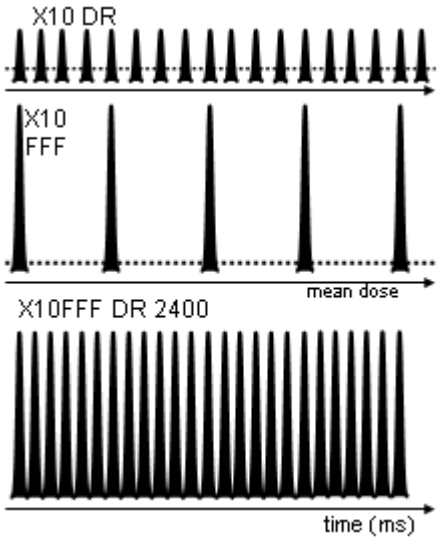


Figure 1: ***beam pulse history***
The pulse pattern of a flattened and a non-flattened beam for the same dose rate (400MU/min) is significantly different. The flattened beam has a high pulse repetition frequency and a low dose per pulse whereas the FFF beam has 4 times more dose per pulse and a lower pulse repetition frequency. Increased dose rate (2400 MU/min) for the FFF beam is achieved by increasing the pulse repetition frequency.

Clonogenic assay
Exponentially growing cells were plated into 30mm Petri dishes. Cells were exposed to 5Gy or 10Gy of irradiation. Following irradiation, cells were incubated for 10-14 days at 37°C in a humidified atmosphere of 5% CO_2 for colony formation. Colonies were fixed with methanol, stained with 1% crystal violet and counted. Colony plating efficiency was calculated as previously described (20).

Statistical analysis
All data are presented as means ± standard deviation (SD). The data was analyzed using the statistical program SPSS version 16.0 (SPSS Inc., Chicago, IL). Differences among the groups were compared by one-way analysis in combination with post hoc Scheffe test. Two-tailed values of $p<0.05$ were considered significant.

Results

Cancer cell survival after irradiation is reduced in a dose rate-dependent manner
To examine the effects of increasing dose-rates on the survival of established brain tumor cell lines, the glioblastoma-derived cell lines T98G and U87-MG were irradiated with either 20, 600 or 2400 MU/min and a dose of 5Gy and 10Gy. Cells irradiated with a dose of 5Gy showed a reduction of clonogenic survival in a dose-rate-dependent fashion. The reduction of cell survival was only significant in cells irradiated with 2400MU/min if compared to cells irradiated with 600MU/min (Fig. 2A). In contrast to the irradiation with 5Gy, clonogenic survival was markedly reduced upon irradiation with a total dose of 10Gy and increasing dose-rates (Fig. 2B) in both cell lines. A 30-fold increase in dose-rate from 20 MU/min to 600

MU/min led to a 2-fold decrease in cell survival in T98G cells irradiated with 10Gy. Further 4-fold increase of dose-rate to 2400 MU/min reduced cell survival by further 1.7 fold. Clonogenic survival of U87-MG was reduced by 0.6 fold and 0.8 fold respectively after irradiation with 10Gy.
We have hypothesized that increasing dose-rates should lead to decreased cell survival in a close-to linear fashion. In contrast to our hypothesis, we observed that clonogenic survival was, although reduced in cells irradiated with a dose-rate of 600 MU/min if compared to the 20 MU/min, much higher in cells irradiated with 2400 MU/min if compared to 600 MU/min.
This effect could arise from an overload of the DNA damage repair machinery by the higher number of pulses-per-minute (Fig. 1), we further investigated if the reduction of cell survival could be a side effect from the beams used in the experiment.

A B

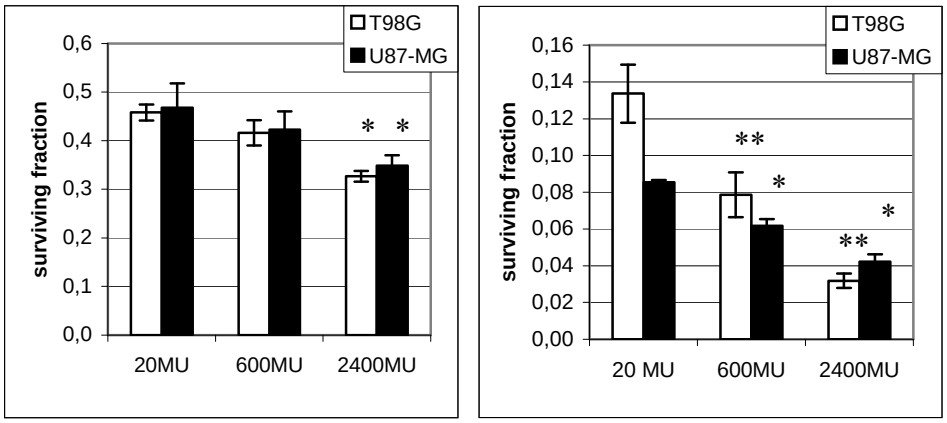
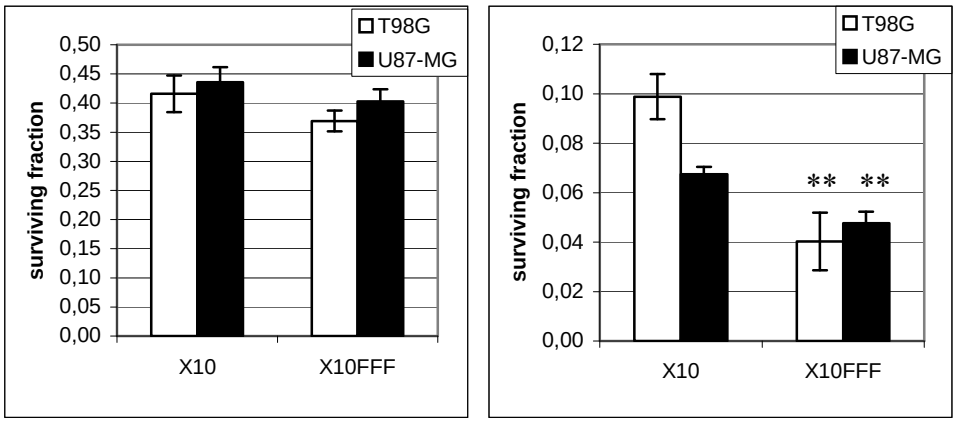


Figure 2: ***IR efficacy is increased with increasing dose rates.***
T98G (empty bars) and U87-MG (filled bars) cells were irradiated with (A) 5Gy (B) 10Gy with dose rates of 20, 600 and 2400MU/min. Clonogenic survival was accessed as a measure for irradiation efficiency. * $p \leq 0.05$; ** $p \leq 0.001$

The use of flattening-filter-free beams increases IR efficacy
Due to the limitations of the TrueBeam linear accelerator, the irradiation using dose-rates of 20 and 600 MU/min were done using the flattened X10 beam while the dose-rate of 2400 MU/min was done using the unflattened X10FF beam. The only dose-rate both beams have available is 400 MU/min. Although both beams deliver the same mean-dose as well as total dose, the dose that is delivered per pulse as well as the number of pulses-per-minute differ significantly (Fig.1). This prompted us to investigate if the reduction of cell survival was also influenced by the beam used for delivery.
T98G and U87-MG cells were irradiated with either the X10 or the X10FFF beam with a dose rate of 400 MU/min. Clonogenic survival assays revealed reduced survival rates after irradiation with the X10FFF beam if compared to the cells irradiated with the X10 beam (Fig. 3A, B). Clonogenic survival of T98G cells in response to irradiation with the X10FFF beam was reduced after an irradiation with 5 Gy (Fig. 3A) but not significantly while it was reduced significantly by 2.4 fold with a dose of 10 Gy (Fig. 3B) if compared to cells irradiated with the X10 beam. The reduction in cell survival after irradiation with 10Gy although still significant was lower in U87-MG.

A B



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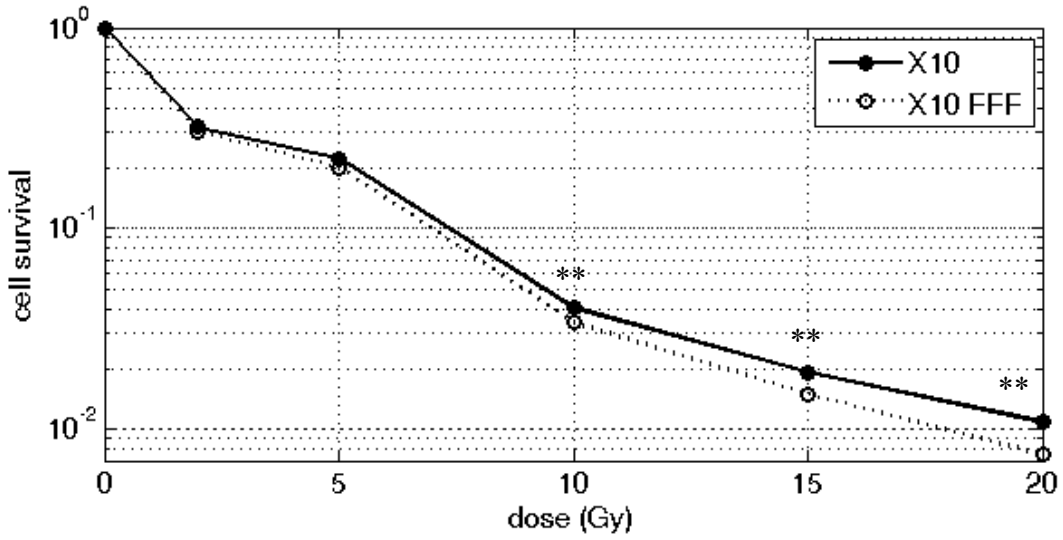

Figure 3: ***Flattening filter-induced reduction in cell death after irradiation.***
T98G (empty bars) and U87-MG (filled bars) cells were irradiated with (A) 5Gy (B) 10Gy with a dose rates of 400Mu/min with either the X10 or the X10FFF beam. (C) Dose response curve of U87-MG cells irradiated with either the X10 (full circles) or X10FFF (empty circles) beam. Clonogenic survival was accessed as a measure for irradiation efficiency. * $p \leq 0{,}05$; ** $p \leq 0{,}001$

In order to test if the observed decrease in clonogenic survival after X10FFF irradiation could be observed over a wider range of doses and if the effect increased with increasing doses, we irradiated U87-MG cells with both the X10 and the X10FFF beam in a dose-response curve with 400 MU/min (Fig. 3C). As suggested by the previous experiments, irradiation with the X10FFF beam reduces clonogenic survival if compared to irradiations using the X10 beam throughout the whole dose-response curve. The survival rates while significantly different after irradiation with higher doses were not significantly different after irradiation doses that are also used for patient treatment.

Increased dose per pulse reduces clonogenic survival

Although both the X10 and the X10FFF beams deliver the dose with a dose rate of 400 MU/min, the dose delivered per pulse as well as the pulses delivered per minute differ between the two beams (Tab. 1). The results obtained might differ between the beams because of the higher dose-per-pulse delivered with the X10FFF. To test that hypothesis, we irradiated T98G (Fig. 4A) and U87-MG (Fig. 4B) cells with 5 Gy and 10 Gy using the X10 and the X10FFF beams in a setup where we reduced the dose per pulse of the X10FFF beam to the level of the X10 beam and the dose was delivered with the same number of pulses per minute. Clonogenic survival assays revealed that clonogenic survival indeed depended on the dose delivered per single pulse. Cells irradiated with the X10FFF beam in the dose-reduction setup showed the same clonogenic survival as cells irradiated with the X10 beam.

A B

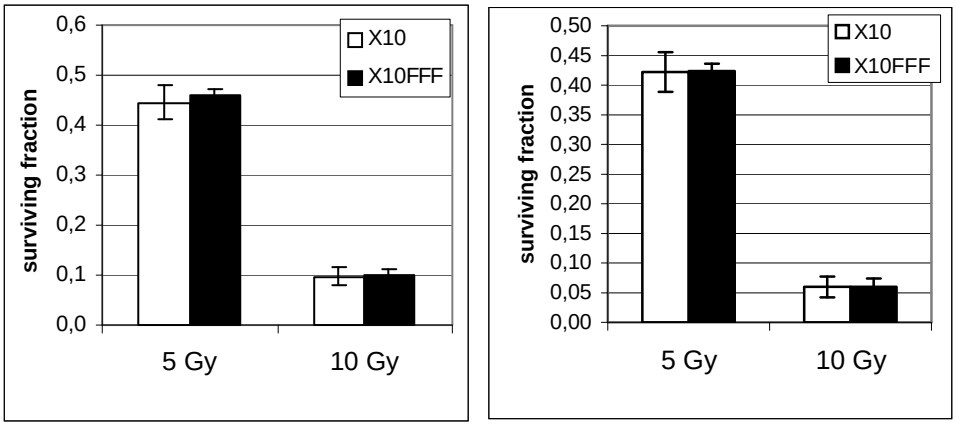

Figure 4: ***Flattening filter-induced reduction in cell death depends on the dose per pulse.***
T98G(A) and U87-MG (B) cells were irradiated with 5Gy and 10Gy with either the X10(empty bars) or the X10FFF beam (filled bars), with reduced dose per pulse. Clonogenic survival was accessed as a measure for irradiation efficiency.

Discussion

The new TrueBeam linear accelerator offers the possibility to irradiate with a higher dose rates than commonly used in radiotherapy. This is achieved by the removal of the flattening filter leading to increased average dose rate and reduced treatment time in patient treatment. Although it has been speculated earlier that cancer cell survival can be reduced with increasing dose-rates (1, 2), few studies have previously aimed to elucidate this hypothesis.
The results presented here show that clonogenic survival is indeed reduced if the total dose is delivered with higher mean-dose rates in a total dose-dependent manner. Due to the limitations of the TrueBeam linear accelerator, the higher dose rates can only be achieved by the use of the unflattened X10FFF beam. This raised the question if and to what extent the change of the beam is responsible for the reduction in clonogenic survival. Indeed, cells irradiated using either the X10 or X10FFF beam show a difference in clonogenic survival which can be explained by the higher dose-per-pulse delivered by the X10FFF.
Further studies are necessary to elucidate the mechanisms by which higher doses delivered per single pulse increase cancer cell death and how normal cell survival is impacted. The delivery of higher doses per pulse might reduce cell survival by an increase in DNA damage induction. Radiation-induced DSBs are the most harmful lesions that arise after irradiation because of its impact on genome stability and cell survival (6). Nevertheless, lesion affecting protein, fatty acids or organelles may play an important role in the induction of cell death

after irradiation. The reduction of cell survival observed whilst reasonably explained with a higher incidence rate of DNA lesion might nevertheless be derived from DNA damage independent pathways. Investigating the role of the unfolded protein response in the endoplasmic reticulum or cytoplasm might therefore give further insight into the pathways induced by changes in dose-rate and dose-per-pulse.
A further increase of the dose delivered per single pulse and the combination of high dose-rates and high doses-per-pulse can possibly reduce cell survival also in the doses used for patient treatment. Additional studies will show how the use of unflattened beam will affect cancer cell survival if the dose is delivered in a fragmentation scheme and if this effect can also be observed in *in vivo* experiments.

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8 Curriculum Vitae

Name	LOHSE
First Name	Ines
Date of Birth	22.08.1981
Nationality	German

Education

1994-2001	Gymnasium Herderschule Lüneburg, Germany Abitur 19.06.2001
10.2001 – 4.2005	University Lübeck, Germany Bachelor of Science 04.04.2005 Thesis: Recombinant production, purification and determination of bioactivity of the hypoxia-induced mitogenic factor.
10.2005 – 03.2007	University Zürich, Switzerland Master of Science 10.04.2007 Thesis: Characterization of novel human Exonuclease 1 interacting proteins
employed as PhD student at the University of Zurich since 01.04.2007	Thesis: Elucidating the role of the hypoxia-protective gene CPT1C in cell biology and carcinogenesis

Occupational Activities

06.-07.2003	Internship: Molecular biology Institute for Physiology, Universität Lübeck, Germany
09.-10.2003	Internship: Mass spectroscopy Wehrwissenschaftliches Institut für Schutztechnologien – ABC-Schutz, Munster, Germany
07.-08.2004	Internship: Virology Wehrwissenschaftliches Institut für Schutztechnologien – ABC-Schutz, Munster, Germany
10.2003-04.2005	Cell biology and Molecular biology student laboratory job Institute for Anesthesiology, Universität Lübeck, Germany
10.2004-02.2005	Teaching assistant in practical courses Institute for Medical Molecular biology, Universität Lübeck, Germany

Publications

1. The CPT1C 5'UTR contains a repressing upstream open reading frame that is regulated by cellular energy availability and AMPK.
   Ines Lohse, Patrick Reilly, Kathrin Zaugg
   Submitted to: Nucleic Acids Research 13.08.2010
2. CPT1C is upregulated by infection with an HIV-based lentiviral RNAi delivery system.
   Ines Lohse, R. Speck, Kathrin Zaugg
   Submitted to: PLOS1 13.08.2010
3. CPT1C: A Putative Regulator of Tumor Growth and Metastasis.
   Manuscript in preparation
4. Effect of high dose per pulse flattening filter free beams on cancer cell survival.
   Manuscript in preparation

Congress participation

Presentations

Lohse I., Feng J., Macedo-Sanchez N., Mak T.W. and Zaugg K. (2009) CPT1C: a Putative Regulator of Tumor Growth and Metabolism. 8th day of Clinical Research, University Hospital Zürich, Zürich Switzerland

Lohse I. (2009) The p53-target CPT1C determines sensitivity to metabolic stress in tumor cells. Weiterbildungsveranstaltung der Klinik für Radio-Onkologie,University Hospital Zürich, Zürich, Switzerland

Lohse I. (2009) Elucidating the role of the hypoxia-protective gene CPT1C in cell biology and carcinogenesis. ZNZ PhD retreat, Rehabilitation Center Valens, Valens, Switzerland

Lohse I., Feng J., Macedo-Sanchez N., Mak T.W. and Zaugg K. (2010) The p53-target CPT1C determines sensitivity to metabolic stress in tumor cells. 15th Annual SASRO Meeting, Inselspital Bern, Bern, Switzerland

Lohse I. (2010) CPT1C: A putative Regulator of Tumor Growth and Migration. Weiterbildungsveranstaltung der Klinik für Radio-Onkologie, University Hopspital Zürich, Zürich, Switzerland

Posters

Reilly P.T., Lohse I., Mak T.W. and Zaugg K. (2008) CPT1C, a stress-inducible protein that regulates cell survival during metabolic stress. 12th Annual SASRO Meeting, Lausanne, Switzerland

Reilly P.T., Lohse I., Mak T.W. and Zaugg K. (2008) p53-dependent regulation of mitochondrial CPT1C determines sensitivity to hypoxia. 7th Day of Clinical Research, University Hospital Zürich, Zürich, Switzerland

Lohse I., Feng J., Macedo-Sanchez N., Mak T.W. and Zaugg K. (2009) CPT1C: a Putative Regulator of Tumor Growth and Metabolism. 8th day of Clinical Research, University Hospital Zürich, Zürich, Switzerland.

Lohse I., Feng J., Macedo-Sanchez N., Mak T.W. and Zaugg K. (2009) CPT1C: a putative regulator of tumor growth and metabolism. ZNZ Symposium, Zürich, Switzerland

Zaugg K., Lohse I., Feng J., Sanchez-Macedo N. and Mak T.W. (2009) CPT1C, a promising anticancer target in the treatment of hypoxic brain tumours. EORTC-NCI-ASCO Annual Meeting on "Molecular Markers in Cancer", Brussels, Netherlands

Zaugg K., Lohse I. and Mak T.W. (2009) Mitochondrial cpt1c determines sensitivity to metabolic stress in tumour cells. 11th International Wolfsberg Meeting on Molecular Radiation Biology / Oncology, Ermatingen, Switzerland

Zaugg K., Lohse I., Feng J., Sanchez-Macedo N. and Mak T.W. (2009) Mitochondrial cpt1c determines sensititivty to metabolic stress in tumour cells. CNIO Cancer conference “The energy of cancer”, Madrid, Spain

Lohse I., Feng J., Tak T.W. and Zaugg K. (2010) CPT1C translation is inhibited by an upstream open reading frame. 9th Day of Clinical Research, University Hospital Zürich, Zürich, Switzerland

Lohse I., Feng J., U.M. Lütolf, Mak T.W. and Zaugg K. (2010) CPT1C: a putative regulator of tumor growth and metabolism. ZNZ Symposium, Zürich, Switzerland

Lohse I., Feng J., U.M. Lütolf, Mak T.W. and Zaugg K. (2010) The CPT1C 5’UTR contains a repressing upstream open reading frame that is regulated by cellular energy availability and AMPK. ZNZ Symposium, Zürich, Switzerland

Zaugg K, Lohse I, Lang S, Hrbacek I, and Lütolf U M (2010) DOSE-RATE EFFECTS OF NOVEL RADIATION TECHNOLOGIES: MOLECULAR ASPECTS ON CANCER CELLS AND NORMAL TISSUE. 29th Annual ESTRO Meeting, Barcelona, Spain

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